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# The impact of asthma, toll-like receptors 7 and 8, genetic and clinical determinants on antiviral immune response

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**Abstract**

People with asthma suffer from worse respiratory infections. The underlying causes have been suggested to arise from type 2-biased inflammation, deficiencies in antiviral immune response, epithelial damage, or a combination of all three. The antiviral immune response is activated by pathogen recognition receptors that include the viral ssRNA detecting toll-like receptor (TLR)7 and its paralogue TLR8. TLR7 induces the production of the antiviral cytokine type I interferon (IFN) that has been reported to be deficient in people with asthma. Additional reports that asthma is associated with reduced TLR7 function and genetic variation in *TLR7/8* gene region warrants investigation of the roles of TLR7/8 in antiviral immune response in healthy people and those with asthma. Three primary aims of this study are:

1. To determine what clinical and immunological parameters are associated with self-reported respiratory infection frequency, the presence of asthma, asthma severity and control, with a specific focus on the role of *TLR7/8* gene expression and function, and the extent to which this differs between women and men.

2. To examine what host and transcriptional variations predict variation in antiviral type I IFN responses *in vitro* and determine how transcriptome patterns vary between individuals with high and low antiviral type I IFN responses.

3. To examine the functional impact that genetic variations in the *TLR7/*8 region has on TLR7/8 gene expression, downstream cytokine production, antiviral immunity, and immune cell counts in peripheral blood.

To address the study aims, I recruited 150 asthma cases and 151 controls, documented self-reported respiratory infection frequency by questionnaire, and measured baseline *TLR7/8* gene expression in whole blood, and cytokine responses using rhinovirus (RV)16-stimulated or TLR7/8-ligand stimulated peripheral blood mononuclear cells (PBMC).

Plasmacytoid dendritic cells (pDC) are of interest to the current study because they are the primary producers of type I IFN and express TLR7. In Chapter 2, we show that measuring *CLEC4C* gene expression provides a useful alternative method for quantifying pDC numbers that may be particularly relevant to large cohort studies where flow cytometry is impractical or inaccessible.

Chapter 3 addresses the first aim of the study and we report that *CLEC4C* gene expression (beta = 0.90, $p$ = 0.01) together with *TLR7* gene expression (beta = -1.13, $p$ = 0.02) are associated with self-reported respiratory infection frequency in men. In women, age (beta = -0.02, $p$ < 0.001) and body mass index (BMI; beta = 0.03, $p$ < 0.001) are associated with respiratory infection frequency. Asthma cases reported significantly more respiratory infections per year than controls (median 3 vs 2, $p$ < 0.001) and reduced *TLR7* gene expression (odds ratio (OR) = 0.12, $p$ = 0.02) and increased BMI (OR = 1.1, $p$ < 0.001). Asthma symptom control was associated with reduced *TLR8* gene expression (beta = -1.4, $p$ = 0.036) and increased BMI (beta = 0.04, $p$ = 0.004), whereas, increased age (OR = 1.0, $p$ = 0.005) and BMI (OR = 1.1, $p$ = 0.004) were associated with asthma severity.

To address the second aim, we sequenced transcriptomes of baseline and RV16-stimulated PBMC samples from subsets of participants with the highest and lowest 15% of IFNα response determined in Chapter 3. In Chapter 4, we report that at baseline in comparison to the low IFNα producers, high IFNα producers have a higher gene expression of innate immune variables including the pDC gene *CLEC4C* and complement genes; and lower gene expression genes, which may be markers of oxidative stress. During antiviral immune response, in comparison to the low IFNα producers, the high IFNα producers express IFNα genes and IFNα response genes at a higher level, as expected, and lower levels of genes linked to antibacterial activity.

For Chapter 5, we genotyped the *TLR7/8* gene region in participants with European ancestry to address aim three. This revealed associations between an asthma risk SNP rs850637 in the *TLR7/8* gene region and basophil count in our study population. Additional analyses using publicly available external databases revealed associations between rs850637 and *TLR7* and *TLR8-AS1* gene expression. *TLR8-AS1* is a type of antisense gene, which are thought to have a function in regulating the gene expression of their complementary gene product. However, we did not find any genetic variants linked to respiratory infection frequency or capacity for IFNα production in vitro.

These findings indicate that multiple risk factors are associated with antiviral immune responses and susceptibility to respiratory infections. These risk factors differ between women and men, with age and BMI prominent in women, whereas innate immune variations were more prominent in men. Decreased TLR7/8 expression in asthma patients may also contribute towards the respiratory infection susceptibility. The asthma-related SNP

rs850637 was associated with lower *TLR7* gene expression and increased basophil count that is associated with asthma inflammation. Considering these findings, TLR7/8 appear to be crucial for the antiviral immune response and asthma inflammation.

**Declaration by author**

This thesis ***is composed of my original work, and contains*** no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, financial support and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my higher degree by research candidature and does not include a substantial part of work that has been submitted ***to qualify for the award of any*** other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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**Publications included in this thesis**

Authors: Liisa Murray, Yang Xi, John W. Upham,

Title: CLEC4C gene expression can be used to quantify circulating plasmacytoid dendritic cells,

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Abstract: Plasmacytoid dendritic cells (pDC) are an important type I interferon producer that play an important role in the first line of host defence during viral infection. Abnormalities in pDC numbers and function have been associated with several health conditions. Quantifying pDC is important for understanding pDC related immune responses in viral infections and other diseases, however the current methods for quantifying pDC using flow cytometry have limited utility in large cohort studies involving multiple centres with limited access to flow cytometry. We reasoned that examining gene expression of the pDC marker C-type lectin domain family 4 member C (CLEC4C, also known as CD303 and BDCA2) in combination with pDC exclusive leukocyte immunoglobulin like receptor A4 (LILRA4, also known as CD85g and ILT7) might provide a more practical method that could be applied to multi-centre studies. Our results show a moderate correlation between pDC numbers measured by surface staining and CLEC4C gene expression in whole blood (rho = 0.39, $p = .037$, as well as a high correlation between CLEC4C gene expression in whole blood and peripheral blood mononuclear cells (rho = 0.79, $p < .001$). LILRA4 gene expression did not provide additional useful information. Our results indicate that measuring CLEC4C gene expression can provide an alternative method for quantifying pDC numbers in human samples.

## Submitted manuscripts included in this thesis

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## Other publications during candidature

Peer-reviewed papers

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Jurak, Lisa M.; Xi, Yang; Landgraf, Megan; Carroll, Melanie L.; Murray, Liisa and Upham, John W. (2018). Interleukin 33 selectively augments rhinovirus-induced type 2 immune responses in asthmatic but not healthy people. Frontiers in Immunology 9 (AUG) 1895.

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Conference abstracts

Murray, L. M., Ferreira, M. A. and Upham, J. W. (2018). What makes a great type I interferon producer? Brisbane Immunology Group retreat, Gold Coast, Australia, 6–7 September 2018.

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**Contributions by others to the thesis**

Primary supervisor Professor John Upham contributed to the conception and design of the project, to the analysis and interpretation of research data and editing of the thesis. Co-supervisor Manuel Ferreira contributed to the conception and design of the project, analysis and interpretation of research data and editing of Chapter 5. Dr Yang Xi measured pDC cell population with FACS in a subset of samples in Chapter 2 and wrote methods for FACS measurements in that chapter. Genome Informatics Group at QIMR Berghofer, Queensland, Australia aligned and mapped RNAseq reads. Dr Alex Cristino from Genomic Medicine group, UQ Diamantina Institute, provided guidance with RNAseq analysis for Chapter 4 and Gayathri Thillaiyampalam from Genomic Medicine group, UQ Diamantina Institute, supervised the transcriptome analysis and performed the GSEA analysis. Dr Stephanie Yerkovich shared her extensive expertise with statistics for the analysis in Chapter 3 and oversaw the linear regression analysis.

**Statement of parts of the thesis submitted to qualify for the award of another degree**

No works submitted towards another degree have been included in this thesis.

**Research Involving Human or Animal Subjects**

All of the components of the research project have been approved by the UQ ethics committee and the Metro South Human Research Ethics committee and performed under research protocol MSHREC:2007/146 and UQ:2008000037.

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**Keywords**

TLR7, TLR8, asthma, common cold, type I interferon, gene variation, gene expression, innate immunity, RNA sequencing, antiviral immune response, immune variation, RNA-seq, transcriptome, IFNα, pDC, respiratory infection, cold frequency

**Australian and New Zealand Standard Research Classifications (ANZSRC)**

ANZSRC code: 060405, Gene Expression, 40%

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FoR code: 0604, Genetics, 40%

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## Dedications

This thesis is dedicated to all those kind-hearted people who volunteered. All the stories they told me about their struggles with asthma and about the people lost to asthma kept me focused and reminded me of the importance of this research.

**Table of Contents**

## List of Tables and Figures

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## List of Abbreviations used in the Document

| Abbreviation | Explanation |
|---|---|
| ACQ | Asthma Control Questionnaire |
| APC | Antigen Presenting Cell |
| B2M | Beta-2-Microglobulin |
| BMI | Body Mass Index |
| cDC | Conventional Dendritic Cell |
| cDNA | Complementary DNA |
| CDHR3 | cadherin related family member 3CDHR3 |
| CLEC4C | C-Type Lectin Domain Family 4 Member C |
| CPM | Counts Per Million |
| DC | Dendritic Cell |
| dsRNA | Double Stranded RNA |
| ELISA | Enzyme-Linked ImmunoSorbent Assay |
| eQTL | Expression Quantitative Trait Loci |
| FACS | Fluorescence-Activated Cell Sorting |
| FC | Fold Change |
| FCS | Foetal Calf Serum |
| FcεRI | IgE Receptor |
| FDR | False Discovery Rate |
| FHS | Framingham Heart Study |
| GINA | Global INitiative For Asthma |
| GO | Gene Ontology |
| GSDMB | Gasdermin-B |
| GSEA | Gene Set Enrichment Analysis |
| GTEx | Genotype Tissue Expression Project |
| GWAS | Genome Wide Association Study |
| HFGP | Human Functional Genomics Project |
| HIV | Human Immunodeficiency Virus |
| HRV | Human Rhinovirus |
| IAV | Influenza Virus A |
| ICAM-1 | Intercellular Adhesion Molecule 1 |
| IFN | Interferon |
| IFNAR | Interferon Alpha/Beta Receptor |
| IFNλR | Interferon Lambda Receptor |
| Ig | Immunoglobulin |
| IKK | Inhibitor of NF-Kb Kinase |
| IL | Interleukin |
| IL18BP | IL18 Binding Protein |
| ILC | Innate Lymphoid Cell |

| | |
|---|---|
| IQR | Interquartile Range |
| IRAK | IL-1R-Associated Kinase |
| IRF | Interferon Regulatory Factor |
| ISG | Interferon Stimulating Gene |
| JNK | Jun Kinase |
| LILRA4 | Leukocyte Immunoglobulin Like Receptor A4 |
| Log | Logarithm |
| MAP3K8 | Mitogen-Activated Protein Kinase Kinase Kinase 8 |
| MDA-5 | Melanoma Differentiation-Associated Gene 5 |
| MDSC | Myeloid Derived Suppressor Cells |
| MyD88 | Myeloid Differentiation Primary Response Protein 88 |
| NEMO | NF-Kb Essential Modulator |
| NES | Normalised Effect Size |
| NFκB | Nuclear Factor Kb |
| NIK | Nfκb -Inducing Kinase |
| NK | Natural Killer Cell |
| OCS | Oral Corticosteroid Medication |
| ORMDL3 | ORM1-Like Protein 3 |
| *P* | P-Value |
| PA | Princess Alexandra |
| PAMP | Pathogen Associated Molecular Pattern |
| PBMC | Peripheral Blood Mononuclear Cell |
| PCA | Principal Component Analysis |
| pDC | Plasmacytoid Dendritic Cell |
| PMNC | Polymorphonuclear Cells |
| PRR | Pathogen Recognition Receptor |
| RIG-1 | Retinoic Acid–Inducible Protein 1 |
| RNA-seq | RNA Sequencing |
| ROS | Reactive Oxygen Species |
| RT-PCR | Reverse Transcription Polymerase Chain Reaction |
| RV | Rhinovirus |
| SLE | Systemic Lupus Erythematosus |
| SNP | Single Nucleotide Polymorphism |
| SOCS | Suppressor Of Cytokine Signalling |
| ssRNA | Single Stranded RNA |
| STAT | Signal Transducer and Activator of Transcription protein |
| TAK1 | TGF-B Activated Kinase 1 |
| TCR | T-Cell Receptor |
| Tfh | T-Follicular Helper cell |
| Th | T Helper cell |
| TLR | Toll-Like Receptor |

| | |
|---|---|
| TNF | Tumour Necrosis Factor |
| TRAF | TNF Receptor-Associated Factor |
| Treg | Regulatory T-Cell |
| TSLP | Thymic Stromal LymphoPoietin |
| UBC | Polyubiquitin-C Precursor |
| UNC93B1 | Unc-93 homolog B1 |
| XCI | X Chromosome Inactivation |

# Chapter 1: Antiviral immunity and asthma

## 1.1. Introduction

Viral respiratory infections are an unavoidable part of everyday life. Though often considered harmless, they disrupt productivity, and in some individuals, these infections may develop into life-threatening illnesses or cause exacerbations of underlying conditions. Asthma patients in particular experience viral respiratory infection-associated complications. This thesis intends to investigate variability in antiviral immune responses in healthy people and those with asthma, with a focus on the role of toll-like receptor (TLR)7 and -8.

The purpose of the current Chapter is to review the current scientific literature concerning these topics. In section 1.2, I first describe the antiviral immune response against rhinovirus, the most common respiratory virus. Next in section 1.3, I review asthma-associated inflammation and the evidence that asthma patients experience worse respiratory infections than their healthy counterparts. Finally in section 1.4, I discuss the current understanding of the immunological and genetic variations influencing antiviral immunity in asthma. It is beyond the scope of this thesis to examine antiviral immunity comprehensively; therefore, the thesis focuses on rhinoviral infections that are the leading cause of respiratory infection-related complications in asthma, and the TLR7/8 mediated immune response. However, for enhanced understanding of those topics, a broader discussion is provided. To expand the knowledge on the topic, we recruited 301 persons for this research study. The research is divided into four parts. The second Chapter describes a methodology to quantify plasmacytoid dendritic cells that are relevant for our understanding of the topic. The methodology was used in the subsequent Chapters. Chapter 3 examines clinical and immunological determinants of respiratory infection frequency and asthma in a large cohort. Some unexpected results from Chapter 3 prompted a comparison of the transcriptomes in individuals whose antiviral immune response was characterised by either high or low interferon alpha production, as detailed in Chapter 4. The fifth Chapter investigates the consequences of genetic variations in TLR7/8. Finally, the results are discussed in Chapter 6 with an emphasis on the central themes of this thesis, and future directions arising from this project.

## 1.2. The antiviral immune response against viral pathogens

The immune response against viral pathogens consists of secreted antimicrobial molecules, efficient detection of pathogen and the subsequent activation of both innate and adaptive arms of immunity. Protection from pathogens is critical for survival; however, excessive immune responses can lead to inflammatory conditions. While all cells respond to viral infection, in the respiratory system bronchial epithelial and immune cells are responsible for their appropriate detection and elimination.

### 1.2.1. Rhinoviruses cause respiratory infections

Many types of viruses cause respiratory infections; most commonly rhinovirus (RV), adenovirus, influenza, parainfluenza, metapneumovirus, bocavirus, respiratory syncytial virus and coronaviruses. Notably, over 90% of the causal viruses are RNA viruses, and of those, RV is detected most commonly (Arden et al. 2006). The RV is known to be the major cause of asthma exacerbations (Denlinger et al. 2011; Miller et al. 2012).

Human rhinoviruses (HRV) are single-stranded RNA (ssRNA) viruses (family *Picornaviridae*) consisting of three different species with HRV-A and HRV-B being the most common and better-known species (Fuchs & Blaas 2012). HRV-C has been identified most recently and has been associated with increased asthma exacerbations in children in comparison to HRV-A and HRV-B (Bizzintino et al. 2011). HRV-A and HRV-B bind to the cell surface receptors intercellular adhesion molecule 1 (ICAM-1), low density lipoprotein receptor or very low density lipoprotein receptors (Greve et al. 1989; Okun et al. 2001) and HRV-C to a cadherin related family member 3 (CDHR3) (Bochkov et al. 2015). Once within the endosome, the virus releases its genome to the cytosol for replication by rupturing the endosome, as shown in Figure 1:1.

Whereas most viruses grow best at the normal body temperature of 37°C, most RV species grow preferentially at 33–35°C, the temperature of the nasal mucosa (Foxman et al. 2015). At 33°C, airway epithelial cells show impaired antiviral responses, with less expression of type I and type III IFNs relative to 37°C (Foxman et al. 2015). This may explain why RV are so adept at causing upper respiratory tract infections. Due to the abundance of ICAM-1 receptor in polarised epithelial cells, the HRV-A and HRV-B are generally found in the respiratory airways (Jakiela, Bogdan et al. 2008), but HRV-A and HRV-C have been shown to primarily infect ciliated epithelial cells (Griggs et al. 2017; Jakiela, B. et al. 2014). This

could explain why RV is unable to infect ciliated nasal epithelial cells that also have low ICAM-1. However, RV can access the basal cells for infection by disrupting the epithelial barrier. RV also upregulates ICAM-1 via a nuclear factor κB (NF-κB)-dependent mechanism, thereby enhancing its capacity to cause infection (Fuchs & Blaas 2012).

Interestingly, atopic patients showed higher levels of ICAM-1 expression in their nasal brushings without infection (Bianco et al. 2000), although in another study bronchial epithelial cells in asthmatic people were found to express less ICAM-1 questioning the role of the receptor in increasing viral titres but perhaps signifying that intracellular mechanisms are more important (Wark et al. 2005). People with asthma have increased numbers of goblet cells, and this may be an additional mechanism promoting RV infection since RV is efficient at replicating in mucus-producing goblet cells (Lachowicz-Scroggins et al. 2010). RV infection does not cause any necrosis to the epithelium. Instead, the symptoms are mainly caused by the host response (Kennedy et al. 2012). The host response to RV will be described in the following section.

### 1.2.2. Virus detection

Antiviral immunity depends firstly on sensing of the virus infection by pathogen recognition receptors (PRRs) that activate inflammatory and antiviral cytokine secretion, which in turn activate antigen presenting cells (APC) to respond and present antigens to the adaptive immune cells. Epithelial cells and innate immune cells such as neutrophils, macrophages and dendritic cells comprise the first line defence against the virus and activate adaptive immunity that subsequently forms targeted immunity and immune memory against the pathogen.

Toll-like receptors (TLRs) are pathogen recognition receptors that have each specialised to recognise a particular pathogen-associated molecular pattern (PAMP) from viruses or bacteria. With such a crucial role, toll-like receptors are highly conserved through evolution (Roach et al. 2013). There are 10 TLRs in humans known today [reviewed for example, by Vidya et al. (2018)]. TLRs recognise a wide variety of PAMPs as their ligands to cover different invading pathogens. TLR1, -2, -4, -5, and -6 receptors are located on the cell surface. TLR1 and -2 recognise bacterial lipoproteins, lipoteichoic acids as well as yeast PAMPs, TLR4 recognises bacterial lipopolysaccharide, TLR6 recognises mycoplasma PAMP, and TLR5 recognises bacterial flagellin. The nucleic acid-detecting TLRs (TLR3, -7,

-8, -9, and -10) are located in endosomes and recognise viral and bacterial nucleic acids. Breakdown of viral material in endosomes exposes the nucleic acid material to which TLRs bind. TLR3 and TLR10 recognise viral double-stranded RNA (dsRNA), TLR9 recognises bacterial DNA, and TLR7 and -8 recognise ssRNA. The function of TLR10 has been elucidated only recently, and it seems to regulate TLR3 function (Lee, SM et al. 2018).

The recognition of ssRNA respiratory viruses, including RV, relies on TLR7 and TLR8. Single-stranded RNA viruses also form dsRNA during their replication process as an intermediate product (Weber et al. 2006), enabling the RV to be recognised by dsRNA receptors TLR3, melanoma differentiation-associated gene 5 (MDA-5) and retinoic acid–inducible protein I (RIG-1) (Parsons, Hsu & Wark 2014). TLR2 is also able to recognise RV capsid proteins (Triantafilou et al. 2011). However, research has suggested that picornaviruses can block RIG-1 action, demonstrating the only known way RV can interfere with interferon (IFN) production (Barral et al. 2009). Despite the reported blocking action, others have shown a function for RIG-1 in RV recognition (Slater et al. 2010). MDA-5 null mice showed reduced IFN response to RV but also reduced airway responsiveness (Wang, Q et al. 2011). This evidence suggests that even though both MDA-5 and TLR7/8, rather than RIG-1, are essential in recognising the nucleic acids from RV and inducing type I and III IFNs, the downstream proinflammatory responses they induce are distinct with MDA-5 driven proinflammatory response probably contributing to airway hyperresponsiveness.

### 1.2.3. Toll-like receptors 7 and 8

The *TLR7* and *TLR8* genes are located on the X chromosome at Xp22 adjacent to each other; both *TLR7* and *TLR8* have three exons, while *TLR8* has two splice variants (Du, X et al. 2000). TLR7 and TLR8 share similarity in their gene sequence and protein structure and are considered close paralogues, derived from the same ancestral gene. TLR7 is expressed constitutively and at a high level in pDC but can also be induced by viral infection in other cell types such as B cells, monocytes, macrophages, natural killer (NK) cells, eosinophils, bronchial epithelial and smooth muscle cells (Drake et al. 2012; Hornung et al. 2002; Shikhagaie et al. 2014; Uehara et al. 2007). In contrast, TLR8 is expressed mainly in monocytes, macrophages and conventional dendritic cells (cDCs)(Hornung et al. 2002; Makni-Maalej et al. 2015).

TLR7/8 are traditionally recognised as viral ssRNA receptors (Heil et al. 2004); however, continued research reveals that RNA degradation products guanosine and uridine activate the receptors in combination with ssRNA (Shibata et al. 2016; Tanji et al. 2015). In addition to viral ssRNA degradation products, DNA derived guanosine activates TLR7, and the receptors also respond to nucleosides from bacteria and protozoa origin (Coch et al. 2019; Gantier et al. 2010; Mancuso et al. 2009). TLR7/8 are proteolytically cleaved to their active forms with the support of a chaperone protein, Unc-93 homolog B1 (UNC93B1), localised in endosomes, where pathogen degradation occurs (Ishii et al. 2014; Lee, BL et al. 2013). Recent advances in understanding the mechanisms regulating TLR7-induced cytokine secretion reveal that TLR7 cytokine secretion exists in two modes, depending on the location with cells. Following TLR7 localisation in endosomes, TLR7 activation induces proinflammatory cytokines such as tumour necrosis factor (TNF) and interleukin (IL)12. In contrast, upon the recruitment of Arl8b and SKIP, TLR7 traffics to the pDC cell periphery to enable type I IFN production (Miyake et al. 2018; Saitoh, S-I et al. 2017).

TLR7 is better studied than TLR8 since for long TLR8 research was hindered by the lack of TLR-specific agonists and antagonists and the assumption that TLR8 is dysfunctional in mice. Currently, a range of agonists are available, and while mice TLR8 function is understood better, it is clear that is functionally distinct from its human homologue as the human TLR8 in mice causes considerable pathology (Snyder et al. 2014). Mouse models reveal that TLR8 depletion in mice results in TLR7 overexpression and oversensitivity to TLR7 agonists, suggesting that TLR8 expression is required to suppress TLR7 expression (Desnues et al. 2014; Pelka et al. 2016; Tran, Manzin-Lorenzi & Santiago-Raber 2015). However, TLR7 and TLR8 are co-expressed in several immune cells in mouse, whereas in humans they are only co-expressed in monocytes and neutrophils, suggesting that the human TLR8 role as a TLR7 regulator may be less significant. Nevertheless, considerable crosstalk exists between individual human TLRs and synergistic effects between TLRs augment the innate immune response (Ghosh et al. 2007). The activation of a combination of TLRs may provide a more tailored response to specific pathogens. For example, the synergistic activation of TLR4 and TLR8 induced augmented IL12 production (Ghosh et al. 2007), and TLR8 has been shown to upregulate TLR2 expression during bacterial infection and downregulate TLR7 and -9 expressions (Cervantes et al. 2011; Cervantes et al. 2012; Wang, J et al. 2006). These studies reinforce the understanding that TLRs have distinct yet complementary roles in pathogen recognition and response.

Even though TLR7 and TLR8 are structurally similar and both recognise similar ligands, it is clear that both serve distinct purposes in antiviral immunity. Not only are they expressed in different cell populations apart from a few cell types but both TLR8 induced cytokines TNF and IL12 participate in activating the adaptive immunity, whereas TLR7 induced IFN production is crucial for innate immune activation. The ability of TLR8 to downregulate TLR7 function, suggests that there is a delicate balance between the effects of the two receptors. The purpose of TLR8 suppressing TLR7 may be to suppress TLR7 induced cytokines in favour of the TLR8 induced cytokines.

### ***TLR7/8 downstream signalling pathways***

To exert their downstream effects, TLRs employ a network of signalling molecules. The TLR7/8 downstream signalling pathways are shown in Figure 1:1. Most TLRs transduce cell signalling via myeloid differentiation primary response protein 88 (MyD88), which is highly conserved and crucial in antiviral immunity. Activation of TLR7 in pDC leads to MyD88 attaching to the receptor and in turn recruiting a signalling complex consisting of IL-1R-associated kinase (IRAK) 1, IRAK4 and interferon regulatory factor (IRF)7. TNF receptor-associated factor (TRAF) 3 and TRAF6 are subsequently recruited to the complex. The inhibitor of NF-κB kinase (IKK)-α phosphorylates IRF7. Also, NF-κB-inducing kinase (NIK), IRAK1 and osteopontin are thought to have a role in its activation. IRF7 is a major hub in RV-induced in vivo models, and it is vital in mediating TLR7 response in plasmacytoid dendritic cells (pDCs) (Bosco et al. 2012). IRF7 is transported to the nucleus where it activates the genes for type I IFN production. IRF7 has been found to have central importance in TLR7 signalling as its depletion results in type I IFN deficiency (Honda et al. 2005). MyD88 is essential for initiating the signalling cascade also in other cell types such as cDCs, B cells and macrophages, where TLR8 is mainly expressed. In the TLR8 signalling pathway, IRAK4 is recruited that then phosphorylates IRAK1 and IRAK2 that activate TRAF6. A complex consisting of TGF-β activated kinase 1 (TAK1), TAK1-binding proteins (TAB)1 and TAB2 is formed that firstly, initiates mitogen-activated kinases (MAPK) pathway resulting in cAMP-responsive element binding protein (CREB) via p38 and Jun kinases (JNKs), secondly, activates IKKβ and NF-kB essential modulator (NEMO) that release NF-κB, and finally, activates mitogen-activated protein kinase kinase kinase 8 (MAP3K8) pathway resulting in AP1. CREB, NF-κB and AP1 translocate in the nucleus and activate proinflammatory cytokines including IL12 and TNF. IRF5 and IRF1 are activated directly by

MyD88 that also regulate gene expression in the nucleus (Blasius & Beutler 2010; Gay et al. 2014).

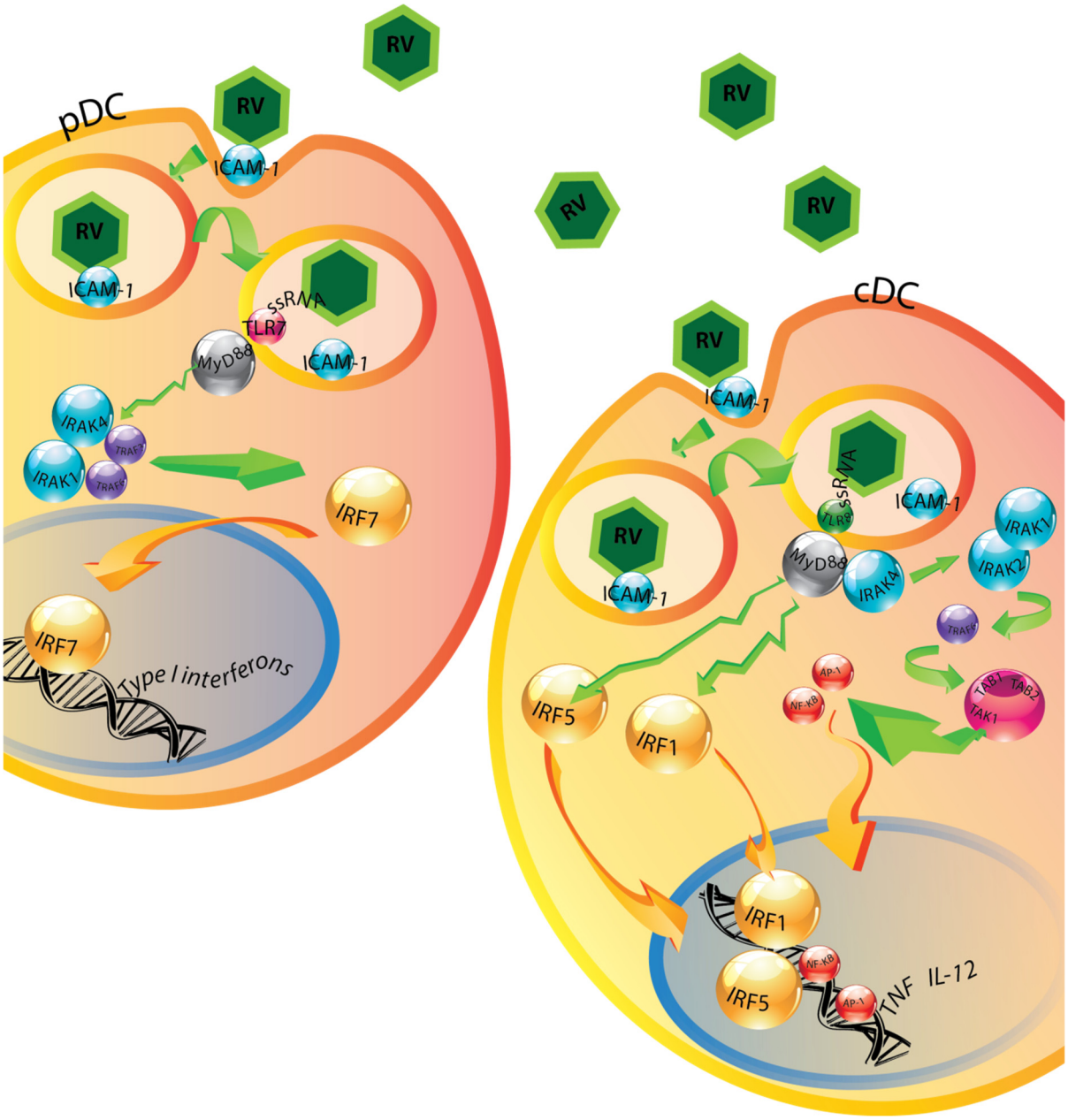


*Figure 1:1 TLR7 and -8 signalling pathways.*
*Rhinovirus (RV) attaches to the intercellular adhesion molecule 1 (ICAM-1) cell surface receptor and enters the plasmacytoid dendritic cell (pDC) (left) by endocytosis. Within the endosome, its ssRNA genome is degraded into oligo-RNA and nucleoside that are TLR7 ligands. Activation of TLR7 recruits a signalling complex that is described in the text in detail. The complex activates IRF7, which acts as a transcription factor for type I interferon production. On the right side, TLR8 in conventional dendritic cell (cDC) detects ssRNA and recruits a signalling complex to release the transcription factors NF-κB and AP1. MyD88 also directly activates transcription factors IRF1 and IRF5 that together with NF-KB and AP1 induce proinflammatory cytokine production. (Original artwork)*

## *Type I interferon induced by TLR7*

Once PRRs detect microbes, the signalling pathways culminate in the production of antiviral cytokines. TLR7 activation can induce either proinflammatory cytokines or type I IFN, one of the most critical types of cytokines in inducing an antiviral state in the organism. IFNα is expressed only upon viral stimulation and otherwise suppressed by the action of IRFs (Levy, Marié & Durbin 2011), whereas IFNβ expression is always maintained at low levels even in the absence of viral infection. The transcriptional regulation of IFNβ is complex as its expression needs to be maintained with a delicate feedback loop.

IFNAR, the common receptor for the type I IFNs, IFNα and IFNβ, consists of IFNAR1 and IFNAR2 and is expressed on all nucleated cells. The type III IFN IFN-λ is produced by many cells, but a component of its receptor IFNλR, IL28Rα, is only expressed in hepatocytes and epithelial cells (Mitchell et al. 2011), conveying a role of epithelial cell protection. IFNAR activation leads to ISGF3 complex activation, which consists of signal transducer and activator of transcription protein (STAT)1, STAT2 and IRF9. The downstream signalling pathways for all IFNs are very similar, but the effect is different for each. The complex initiates IFN-stimulated gene (ISG) activation. Modern computational approaches have enabled the recognition of thousands of differentially regulated genes upon IFN stimulation (Green, R, Ireton & Gale 2018). Core ISGs lead to the induction of an antiviral state in cells, dendritic cell maturation, adaptive immune response activation, and secretion of chemokines to recruit lymphocytes and monocytes to the site (Raftery & Stevenson 2017; Schneider, Chevillotte & Rice 2014).

During viral infection, type I IFN production in pDCs is significantly enhanced with pDC clustering, where cell-cell contact triggers a paracrine loop amplifying type I IFN production (Kim et al. 2014; Wimmers et al. 2018). A detailed study by Saitoh, S-I et al. (2017), reveals that TLR7 activation induces proinflammatory cytokines in the absence of pDC clustering and further evidence of feedback loop between the proinflammatory cytokines (TNF and IL12) and type I IFN suggests that the TLR7-induced proinflammatory cytokine production may act in priming pDC for robust type I IFN responses (Gautier et al. 2005; Lee, J et al. 2015). This mechanism presents just one regulatory mechanism for IFN production, in addition to the activation of ISGs, of which regulatory proteins suppressor of cytokine signalling (SOCS) in particular have a role in regulating IFN production.

### ***Proinflammatory cytokines induced by TLR8***

TLR8 activation induces proinflammatory cytokines TNF, IL12, and to a lesser extent, CCL3 (also known as MIP-1α), but less type I IFN synthesis than TLR7 (Gorden et al. 2005). The difference in the cytokine profile expressed between TLR7 and TLR8 is due to their expression in different cell populations and different cell signalling network induced as described above. As the name suggests, tumour necrosis factor (TNF) was originally found to induce cell death through activating apoptotic pathways. In an antiviral response, apoptosis in infected cells prevents the pathogen from replicating using the transcription machinery of the host cell; therefore, is a crucial component of antiviral response. Recently, the roles of TNF have been expanded beyond cell death and functions to support both innate and adaptive immune responses (Yi et al. 2018). TNF induces the secretion of some other cytokines such as IL1 and IL18 and in the respiratory epithelium; TNF also induces β-defensin secretion, which is an antiviral peptide. Many viruses have developed mechanisms to prevent TNF mediated signalling as a defence, which demonstrates its importance to antiviral immunity (Waters, Pober & Bradley 2013). To support adaptive immunity, TNF co-stimulates CD4+ and CD8+ T-cell activation and differentiation through the TNF-family receptor Fas (Yi et al. 2018).

Similarly, IL12 is also induced by TLR8 agonists. Its primary function is to direct T-helper cell differentiation towards a Th1 response. IL12 induces the transcription factor T-bet, which stimulates naïve CD4+ T cells to differentiate into Th1 cells and produce IFNγ (Yu et al. 2015). CCL3 is a proinflammatory and chemotactic factor for recruitment of IFNγ-activated neutrophils and CCR1-expressing eosinophils (Maurer & von Stebut 2004). IL18 and IFNγ are induction are mediated by TNF and IL12 secretion, and together, they activate Th1 differentiation and enhance NK-cell cytotoxicity (Akdis et al. 2016).

### 1.2.4. Innate immune response

The antiviral innate immune response is the first non-specific response of the immune system against an invading pathogen before the pathogen-specific adaptive response is activated. The first defence involves baseline levels of antimicrobial peptides that may have antiviral properties also. Epithelial cells secrete antimicrobial peptides upon TLR activation, a mechanism that likely preserves the commensal pathogen colonies instead of activating the cellular responses (Uehara et al. 2007). The complement system consists of secreted

peptides that tag both bacteria and viruses for degradation and complement cellular immune responses in several ways (Hajishengallis et al. 2017).

The next level of defence involves the recognition of pathogen degradation products, which triggers cellular responses. As described above for TLR7/8, PRR activation induces secretion of antiviral and proinflammatory cytokines and chemokines for the recruitment of other cell types. Although some proinflammatory cytokines support antiviral function, the role of IFNs is the most central in antiviral immunity as seen in those rare individuals who are markedly deficient in type I IFN production and highly vulnerable to lethal virus infections (Duncan et al. 2015; Taft & Bogunovic 2018).

IFNs activate professional APCs such as dendritic cells and macrophages, but also other cells such as neutrophils and eosinophils have been shown to contribute to antigen uptake and presentation in viral infections (Hufford et al. 2012; Sabogal Piñeros et al. 2019; Su et al. 2015). They secrete chemicals to defend against the pathogen and chemokines to attract adaptive immune cells as a second line of defence. Neutrophils secrete extracellular traps and antiviral factors to attack against the pathogen (Saitoh, T et al. 2012). Eosinophils secrete RNA degrading ribonucleases, which partly explains their role in the ssRNA RV infection (Gupta et al. 2013). Other eosinophilic defence mechanisms include the secretion extracellular traps and reactive oxygen species species (Kovacs et al. 2014; Silveira et al. 2019).

Although pDC form only a fraction of immune cells, they are crucial in antiviral immunity as shown with pDC depletion experiments (Xi et al. 2017). Our laboratory has previously shown that pDCs comprise more than 90% of all IFNα producing cells, with monocytes 10% and other dendritic cells less than 1% (Xi et al. 2015). In support, animal models further show that pDC depletion diminishes type I IFN production and causes considerable viral pathogenicity (Swiecki, M. et al. 2010). As the primary producer of type I IFN, pDCs are abundant in TLR7 and TLR9 expression to respond to invading viruses. The antiviral type I IFN response in pDCs has been shown to be dependent on TLR7 function (Davidson et al. 2011; Kaiko et al. 2013). The type I IFN production induces an innate immune response, and as a dendritic cell, pDCs are further able to activate adaptive immunity by presenting antigens to naïve CD4+ and CD8+ cells, playing a crucial regulative role between the innate and adaptive immunities (Lynch et al. 2014).

### 1.2.5. Adaptive immune response

The activation of adaptive immunity is vital for long-lasting protection against pathogens. The focus of this thesis is in the innate immune responses, but to understand the complete antiviral immune response, adaptive immune responses are briefly discussed here.

IFNs exhibit both direct and indirect effects of activating the T-cell immune response. Those mechanisms are summarised here and reviewed in full detail in Crouse, Kalinke and Oxenius (2015). The indirect mechanism is mediated by type I IFN-activated APCs. The mature APCs secrete chemokines to recruit adaptive immune cells. APCs express MHC molecules and co-stimulatory molecules that, together with the presented processed pathogen antigens, activate the adaptive immune cells to produce pathogen-specific effector and memory cells. APCs also secrete cytokines IL15 and IL7 that support T-cell proliferation, IFNγ that supports Th1-cell differentiation and IL12 that supports survival and differentiation of activated cells. The direct effects of type I IFN on T-cells depend on the activation sequence of IFNAR and T-cell receptor (TCR). If TCR activation precedes IFNAR activation, T-cell survival, differentiation and protection from NK-cell mediated cytotoxicity prevail, whereas, if IFNAR activation precedes TCR activation, T-cells activate opposite cellular programmes and become susceptible to NK-cell destruction.

CD4+ T-helper cells can differentiate into Th1, Th2, Th17, Treg, Tfh, or the less well-known Th22, Th3, Tr1 or Th9 types. Th1 type is predominantly responsible for antiviral immune response and induces IL2, IFNγ and TNF cytokines. Th1 differentiation is initiated with the main transcription factor T-bet along with several other transcription factors, while the Th2 transcription factor GATA-2 is suppressed (Saravia, Chapman & Chi 2019). The antiviral Th1 cells help activate humoral B-cell responses with IFNγ secretion, promote cytotoxic and memory CD8+ T-cell responses with IL2 secretion, support APC activation and also directly induce an antiviral state in cells and kill infected cells. After viral infection, a small population of Th1 cells differentiate to memory cells. (Swain, McKinstry & Strutt 2012). Evidence with RV and influenza infection suggests that CD4+ memory cell have a direct antiviral function, independent of their helper function (Muehling et al. 2016; Teijaro et al. 2010).

Notably, CD4+ T cells have also been found to express TLR7, and TLR7 activation was shown to induce anergy in those cells. Deactivation of TLR7 produced better defence against human immunodeficiency virus (HIV), indicating that while TLR7 is critical in acute

infection inducing innate immunity, in chronic infections its role is more detrimental if it prevents proper antigen recognition by CD4+ T cells (Dominguez-Villar et al. 2015).

Most recently, an important innate lymphoid cell (ILC) population has been identified. Research on ILCs has expanded rapidly and advanced the knowledge of ILC differentiation into type 1, 2, and 3 ILCs as well as cytotoxic NK-cells (Lim et al. 2017). The distinctive functions of ILC1, -2, and -3 types align with the functions of their adaptive T-helper cell counterparts and while it has become apparent that while they cannot exhibit specialised adaptive immune function, they support the Th cells in maintaining their respective cytokine milieu (Mjösberg & Spits 2016). An equally important function is the direct interactions ILCs exert on other immune and non-immune cells such as activating DCs and regulating epithelial function (Mjösberg & Spits 2016).

## 1.3. Asthma patients suffer from severe respiratory infections

Asthma patients are burdened with severe respiratory infections, including those caused by an RV. The type 2 dominant immune response in early-onset allergic asthma and the 'normal' Th1 antiviral immune response are traditionally thought to antagonise each other, though this is probably an oversimplification, as discussed further in sections 1.4.1 and 1.4.2. In this section, asthma and severe respiratory infections are discussed.

### 1.3.1. The definition of asthma

Asthma is a chronic disease of the airways. Clinical symptoms, which include wheezing, tightness in the chest, difficulty breathing and night time coughing, can be brought on by various triggers. In Australia, 1 in 10 people has asthma (Australian Centre for Asthma Monitoring 2011), with many individuals with asthma experiencing a reduced quality of life, loss of productivity at work or studies, and the financial consequences of the ongoing healthcare costs associated with this disease. In Australia, asthma causes the death of just over 400 people each year (Poulos et al. 2014).

The three cardinal features of asthma are variable airway obstruction, airway hyperresponsiveness and airway inflammation. The airways of people with asthma exhibit an exaggerated contractile response to inhaled stimuli such as allergens or smoke (Australia. 2015; McCarty & Ferguson 2014). Airway hyperresponsiveness facilitates bronchoconstriction, which is responsible for most of the physiological changes that cause asthma symptoms. Airway inflammation leads to more severe airway hyperresponsiveness (McCarty & Ferguson 2014). For example, allergen exposure stimulates the release of a variety of inflammatory mediators that cause airway inflammation and increase mucus production and bronchoconstriction by activating airway smooth muscle. Persistent asthma results in more permanent changes to airway structure called airway remodelling (Lambrecht & Hammad 2012).

### 1.3.2. Different asthma phenotypes

Different phenotypes of asthma have long been recognised, and multiple attempts have been made to categorise asthma based on properties such as triggers, age of onset, physiological features, responsiveness to treatment, and the dominant airway inflammatory cell type (Corren 2013; Hekking & Bel 2014). The literature recognises allergic asthma as

the most common asthma type, with an onset, typically in childhood. It is characterised by eosinophilia and allergen-specific immunoglobulin (Ig)E and is exacerbated by allergic triggers. Asthma symptoms are controlled with medication and asthma treatment intensity, responsiveness to treatment, and control of symptoms with treatment present additional asthma phenotype considerations.

Asthmatic airway inflammation varies, and this has given rise to four common inflammatory phenotypes, namely eosinophilic asthma, neutrophilic asthma, mixed granulocytic asthma and paucigranulocytic asthma (Simpson et al. 2006). The most common phenotype is known as eosinophilic asthma and is characterised by a sputum eosinophil concentration of ≥ 1.01% in the presence of asthma symptoms and airway hyperresponsiveness (Simpson et al. 2006). Eosinophilic airway inflammation in asthma occurs as a result of $CD4^+$ T helper cell differentiation skewing towards a type 2 response, which is discussed in the next section.

Neutrophilic asthma is characterised by an increased proportion of neutrophils in the airways, in the presence of asthma symptoms and airway hyperresponsiveness. Individuals with neutrophilic asthma tend to be non-atopic and female, with a later onset of disease (Green, RH et al. 2002). Neutrophilic asthma is triggered by environmental exposures such as ozone, bacterial endotoxins and viruses (Douwes et al. 2002). Although inflammation of the airways is a prominent feature of neutrophilic asthma, the cytokine response differs from that of eosinophilic asthma. In some asthmatics, neither an excess of neutrophils nor eosinophils are present in sputum despite the persistence of asthma symptoms forming the paucigranulocytic asthma group. The paucigranulocytic asthma type has been described as the most benign asthma type (Demarche et al. 2016; Ntontsi et al. 2017). In contrast, mixed granulocytic asthma inflammation is characterised by mixed eosinophilic and neutrophilic inflammation and shares features of both types (Ntontsi et al. 2017).

With the advent of more sophisticated tools, asthma classification has moved towards the integration of clinical features, inflammation types and molecular properties to define more accurate phenotypes termed as endotypes. Classifying asthma type in more detail provides the prospect of identifying the endotypes most at risk of infection-related complications and tailoring asthma treatment to suit the patient better (Deliu et al. 2016).

### 1.3.3. Type 2 dominant inflammation in asthma

While Th1 responses are crucial for viral defence, Th2 responses are crucial against parasitic pathogens. Differentiation to Th1 or Th2 is a highly polarised mechanism, as is evident from the crossregulation of activating cytokines and transcription factors observed in Th1 and Th2 responses (Saravia, Chapman & Chi 2019). Th2 responses can be abnormally activated by allergens in asthma, as is explained in Figure 1:2. In the lung tissue, the allergens detected by APCs such as DCs, macrophages and monocytes activate type 2 immune responses. Initially, this was thought to involve only conventional T- helper cells (Th2), but more recent studies have highlighted the importance of ILCs, so type 2 immune response or type 2 inflammation are now the preferred terms (Mjösberg & Spits 2016). Currently, it is known that epithelial cells are also directly activated by allergens and produce IL33, IL25 and thymic stromal lymphopoietin (TSLP) cytokines (Angkasekwinai et al. 2007; Kurowska-Stolarska et al. 2008; Rochman et al. 2007). These epithelial-induced cytokines act on naïve T-cells and type 2 innate lymphoid cells (ILC2) to induce type 2 cytokine production.

Type 2-dominant asthma inflammation is a consequence of Th2 polarisation and ILC2-dependent processes. Type 2 cytokines IL13, IL4, IL5 and IL9, recruit eosinophils, induce B-cell switching to produce allergen-specific IgE and activate mast cells. Granulocyte infiltration in the lung causes many of the symptoms experienced by patients. In type 2 inflammation, eosinophils, mast cells and basophils dominate and contribute to the airway inflammation, hyperresponsiveness, mucus secretion and epithelial cell lining damage. In addition to allergens, airway irritants can act via the innate ILC2 cells in asthma; these produce IL5 and IL13 in the absence of adaptive immunity (Hirose et al. 2017; Lambrecht & Hammad 2014; Scanlon & McKenzie 2012).

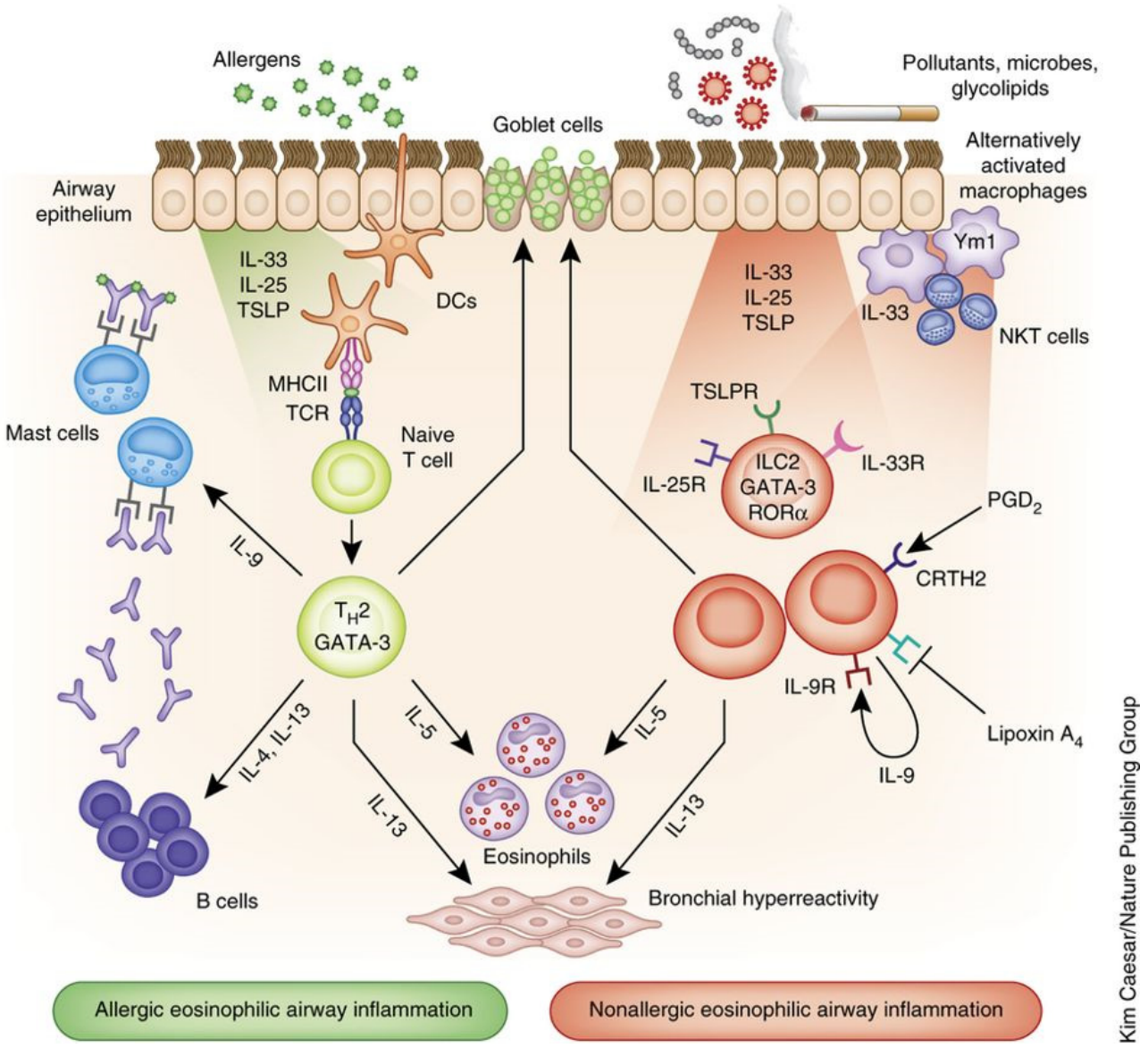


*Figure 1:2 Allergic and non-allergic airway inflammation.*
*In allergic airway inflammation, allergen stimulates epithelial cells to secrete IL33, TSLP, and IL25 and DCs to activate T-cells. Activated Th2 cells recruit eosinophils and mast cells, activate B-cell class switching and cause bronchial hyperreactivity. In the presence of non-allergenic stimuli, IL33, TSLP, and IL25 are secreted by eosinophils and macrophages but activate ILC2 to secrete type 2 cytokines. Reprinted by permission from Springer Nature (Lambrecht & Hammad 2014).*

### 1.3.4. Severe respiratory infections and risk of exacerbations in asthma

A comparative longitudinal study of couples where one partner was asthmatic, found that the asthmatic partners suffered from more severe lower respiratory infections caused by RV than the healthy partners, whereas upper respiratory infections occurred with similar frequency in both groups (Corne et al. 2002). A smaller scale study found similar results with experimental RV infection: asthma was associated with lower respiratory tract infection

rather than being limited to upper respiratory tract infection as seen in healthy people. Interestingly, there was a correlation between the viral load and the severity of symptoms in asthmatic people but not in healthy controls (Message et al. 2008). Increased RV load in asthma patients has not been replicated by all studies (DeMore et al. 2009; Denlinger et al. 2011; Kennedy et al. 2014) but may be associated with co-infection with pathogenic bacteria (Kloepfer et al. 2014). However, influenza infections seem consistently severe in asthma (Kloepfer et al. 2012; Santillan Salas et al. 2013). Because many of the experimental infection studies include only mild asthma patients, it is possible that the increased infection severity might only be seen in severe asthma patients or with a large amount of virus. The exposure to allergens seems to also complicate the defence against a virus. Total IgE was shown to positively correlate with worse respiratory infection symptoms (Zambrano et al. 2003).

In addition to severe cold symptoms, also asthma symptoms are aggravated during respiratory infections. Viral infections were recognised as a major cause of asthma exacerbations over 20 years ago (Nicholson, Kent & Ireland 1993). RV infection was found to increase the risk of an asthma exacerbation almost two-fold in children (Miller et al. 2012). Co-exposure to allergens together with a virus may further increase the likelihood for acute asthma exacerbations resulting in hospitalisations, compared to just allergen or virus alone (Murray, CS et al. 2006). Respiratory infection is likely to cause excessive inflammation in the setting of the already inflamed allergic asthmatic airways.

One explanation for the difference is altered ability to clear the viral infection. Bronchial epithelial cells from asthmatic people are compromised in their ability to undergo apoptosis (Wark et al. 2005). Because early apoptosis is important for clearing viruses, this is a potential mechanism by which asthmatics cannot clear viruses as efficiently as healthy people. Physiological properties of the asthmatic airways could contribute to infection susceptibility as asthmatic airways are chronically inflamed and primed for bronchoconstriction upon a variety of external stimuli. Since the asthmatic airways are already damaged from a constant state of inflammation, in theory, this could make it easier for pathogens to infect. The type 2 cytokine IL13 increases the numbers of mucus-producing goblet cells in asthma, and it was shown that RV preferentially infects goblet cells, boosting RV infection opportunity in asthmatic airways (Lachowicz-Scroggins et al. 2010). While

these mechanisms may contribute to the worse infection in asthma patients, deficiencies in the antiviral immune response are also likely to contribute and are discussed next.

## 1.4. Is there a deficient antiviral immune response in asthma?

Asthma has been found to be a risk factor for several microbial infections comprehensively reviewed by Juhn (2014). The authors also discuss how asthma treatment plans should include a focus on infection prevention and argue that asthma is an immunodeficiency condition. In support of this idea, a recent study with over 100,000 participants revealed that people with asthma have a hazard ratio of 1.65 for infection-related hospitalisation (including non-respiratory infections) compared to people with no atopic conditions (Helby et al. 2017). While many questions remain, much progress has been made in previous years to understand how asthma patients are deficient in their antiviral immune responses.

### 1.4.1. T-helper cell response imbalance

The type 1 response is necessary for efficient antiviral immune response, whereas in asthma, the airway inflammation is usually characterised by a type 2 response. The bias towards type 2 response in asthmatics extends to antiviral response as well: while healthy people have a type 1 dominated response, asthmatics seem to have a more type 2 dominated response against viral infections (Brooks et al. 2003; Message et al. 2008). The Th1 adaptive immune response induced by IL10 and IFNγ in experimental RV infection was found to be associated with milder infection in healthy controls, whereas secretion of Th2 cytokines IL4, -5, -13 were associated with asthma and more severe infection as measured from sputum, blood and bronchial lavage (Message et al. 2008). We have shown that the type 2-skewing cytokine IL33 induces type 2-dominant RV response in asthmatics but not in healthy people (Jurak et al. 2018), suggesting that the allergen-induced IL33 production in the lung can impair sufficient Th1 polarisation for antiviral immune response. Type I IFNs are able to suppress type 2 immune response and upregulate type 1 response, and the downregulation of type 2 responses in ILC2 further reduces airway hyperreactivity, a mechanism which has been found to be mediated by the TLR7/8-dependent type I IFN production in pDC (Duerr et al. 2015; Maazi et al. 2018; Pritchard, Carroll, et al. 2012). The next section reviews the literature on the current understanding of IFN deficiency in asthma, and how this may contribute to imbalances in type 1/type 2 immune responses.

### 1.4.2. How consistent is the evidence for interferon deficiency in asthma?

Due to the central role of IFN in antiviral immune response, much of the research effort has focused on examining IFN production in asthma patients. IFNα production has been shown

to correlate with viral load, providing evidence that low IFN production can cause higher viral load and more severe infection (Contoli et al. 2006; Edwards, MR et al. 2013). Several studies to date have reported type I IFN deficiency in response to virus across various age groups in airway epithelial cells of asthma patients (Baraldo et al. 2012; Edwards, MR et al. 2013; Sykes et al. 2012; Wark et al. 2009; Wark et al. 2005; Zhu et al. 2019) and in circulating blood leukocytes (Bufe et al. 2002; Durrani et al. 2012; Gehlhar et al. 2006; Iikura et al. 2011; Pritchard et al. 2014). Additionally, type III IFN, the prominent IFN produced by the epithelium, has also been reported to be lower in asthma patients (Baraldo et al. 2012; Contoli et al. 2006; Edwards, MR et al. 2013). However, not all studies have confirmed IFN deficiency in asthma (Lopez-Souza et al. 2009; Patel et al. 2014; Sykes et al. 2012; Sykes et al. 2014).

The discrepancy between the studies suggests that IFN deficiency may be associated with a specific asthma phenotype, and many of these studies have, therefore considered the heterogeneity of asthma. Some report that proper asthma symptom control may preserve the IFN response in asthmatics (Sykes et al. 2014) and others report that IFN deficiency is only observed in severe asthma (Edwards, MR et al. 2013). A limitation for considering the association with specific asthma phenotypes is that many of the aforementioned studies included only mild individuals. However, they show that inhaled steroid medications, taken to moderate and severe asthma symptoms, do not seem to influence IFN production (Contoli et al. 2006; Wark et al. 2005). The role of allergies seems important as total IgE has been shown to inversely correlate with IFN production (Baraldo et al. 2012; Gill et al. 2010), although also non-atopic asthmatic children had decreased IFN production compared to non-atopic non-asthmatic children (Baraldo et al. 2012). Interestingly, a study in older adults found that deficient IFNα production was associated with poorly controlled neutrophilic asthma, but was not associated with atopy or eosinophilic asthma (Simpson et al. 2016). The association between neutrophilic asthma and IFNα production is somewhat surprising considering that counterregulation between IFN production and Th2 immunity is strongly supported in the literature (Koltsida et al. 2011; Pritchard, White, et al. 2012).

Together, the studies examining viral load described in section 1.3.4 and examining IFN production described here suggest that deficiencies in antiviral immune responses are not universal across all asthma patients but may only affect only a subgroup of patients or affect different asthma phenotypes at different degrees. A study from last year employed machine

learning to identify cytokine response profiles that are associated with asthma in a birth cohort of 300 children, studied at the age of 11 years (Custovic et al. 2018). The cluster at the highest risk of having asthma in the early years of life had the lowest IFN response, a finding which supports the notion that asthma is associated with IFN deficiency. However, the cluster with the highest IFN response to RV was similarly associated with asthma, but only when diagnosed in later childhood. The work of Custovic et al. (2018), supports the view that only a subgroup of asthmatics are susceptible to IFN deficiency as asthmatic children were present in each cytokine response cluster. Sample size is a significant limitation to some of these studies, for example Lopez-Souza et al. (2009) reported no difference in IFN synthesis between just six asthma cases and five controls. Given that the research field is yet to agree what asthma phenotype is most closely linked to antiviral type I IFN deficiency, some of these small studies are likely underpowered to show immune deficiency or phenotype driving the deficiency, and further investigations are needed in much larger studies.

Interestedly, the number of type I IFN producing pDCs has been found increased in asthmatic adults, whereas their number was found reduced in preschool children who subsequently developed asthma (Silver et al. 2009; Spears et al. 2011; Upham et al. 2009). The theory of immunodeficiency in asthma, proposed by Juhn 2014, is in line with this finding as pDCs in asthmatics have been found deficient in producing antiviral IFNα in response to an influenza virus, partly mediated by IgE crosslinking of IgE receptors on the surface of pDC (Gill et al. 2010). Therefore, it seems plausible to propose that asthmatic adults have a feedback mechanism in place for increasing the number of pDC in response to viral infections in order to ensure sufficient type I IFN production. Such a mechanism might not be sufficiently developed in young children, such that a developmental delay in pDC maturation or attainment of maximal type I IFN synthesis might predispose to early and severe respiratory viral infections and subsequent asthma development. A study demonstrated that type I IFN production induces pDC apoptosis in the spleen, confirming a feedback mechanism between pDC numbers and amount of type I IFN (Swiecki, M. et al. 2011). Furthermore, pDCs were found to downregulate type 2 cytokine response after RV stimulation (Pritchard, Carroll, et al. 2012). Since different types of IFN have been shown to be low in asthmatics upon viral stimulation, absence of negative type 2 regulation from IFN can further contribute towards the type 2 cytokine biased response.

### 1.4.3. Are TLR7 and TLR8 associated with asthma?

Exogenous IFNβ is able to rescue the normal antiviral response in asthmatic bronchial epithelial cells; hence, the associated immune defect most likely lies upstream from type I IFN synthesis (Cakebread et al. 2011). Thus, the cause for low type I IFN production is likely to involve variations in the ability of viruses to enter cells, the ability to detect viral nucleic acids within cells, or the ability to induce IFN gene transcription. Deficient TLR7 function would explain inadequate type I IFN production and susceptibility to viral infections in asthma. Compromised TLR8 function could also prevent proper Th1 induction and contribute to Th2 bias in asthma.

Around the same time as IFN deficiency was first identified in asthma (Bufe et al. 2002), a Danish research group identified the X-chromosome region that contains *TLR7* and *TLR8* as a candidate region for asthma susceptibility (Haagerup et al. 2002), subsequently confirmed in another cohort (Brasch-Andersen et al. 2008; Haagerup et al. 2004) with the association restricted to *TLR7* SNP rs179008 and *TLR8* SNP rs2407992 (Moller-Larsen et al. 2008). *TLR7/8* SNPs were also associated with asthma in a Chinese Han population (Zhang, Q et al. 2015), in Finnish children after contracting bronchiolitis as infants (Törmänen et al. 2017), and in Brazilian families (Genov et al. 2014). Recently, a SNP rs850637 in the *TLR7/8* gene region was identified as an asthma risk gene in a GWAS study (Ferreira et al. 2019). Several associations have also been reported between allergic rhinitis and *TLR7/8* in Swedish and Chinese populations (Nilsson et al. 2012) and a Swedish population (Henmyr et al. 2016). Together, these studies indicate genetic alterations in the *TLR7/8* genes in asthma patients, with the potential to affect their function.

Indeed, impaired TLR7 function has been reported in asthma patients. Plasmacytoid DC and alveolar macrophages from asthma patients were shown to express less TLR7 gene (Pritchard et al. 2014; Rupani et al. 2016) and protein (Rupani et al. 2016) after RV activation. TLR7 stimulation with a specific agonist resulted in reduced gene expression of several ISGs in peripheral mononuclear cells (PBMC) from asthma patients (Roponen et al. 2010). Shikhagaie et al. (2014) showed that asthmatic patients had reduced expression of TLR7 in bronchial basal, ciliated and goblet epithelial cells together with leukocytes such as pDC, CD8+ and CD4+ T lymphocytes, EG2+ eosinophils and mast cells. The reduction in TLR7 expression correlated with asthma severity. Shikhagaie and colleagues (2014) also found overexpression in neural cells, which is intriguing.

Two of the studies included mild-to-moderate atopic asthmatic patients (Pritchard et al. 2014; Roponen et al. 2010), but one study reporting changes in TLR7 expression and function included both atopic and non-atopic severe asthma patients (Rupani et al. 2016) and found that *TLR7* gene expression was similar between atopic and non-atopic asthmatics. Roponen et al. (2010) found that the TLR7 impairment was seen only in atopic asthmatics and not in atopic non-asthmatics or non-atopic asthmatics, together suggesting that the TLR7 impairment is independent of atopy status. Instead, Rupani et al. (2016) showed that *TLR7* gene expression correlates inversely with asthma control questionnaire (ACQ) score and the number of asthma exacerbations suffered in the past 12 months, suggesting either that poorly controlled airway inflammation might reduce TLR7 function, or that impaired TLR7 expression or function predisposes to repeated virus infections, poor asthma control and recurrent asthma exacerbations. While asthma control is similar between different asthma inflammatory phenotypes (Ntontsi et al. 2017), reduced TLR7 expression was seen only in eosinophilic phenotype in another study (Hatchwell et al. 2015). The conflicting reports could be due to the different cell sampled, as Hatchwell et al. (2015) studied endobronchial biopsies.

Even though *TLR8* SNPs have been associated with asthma, research on TLR8 in asthma is scarce (Pritchard et al. 2014; Sykes et al. 2013). TLR7 seems to be the popular sibling due to its ability to induce type I IFN, whereas the role of TLR8 lies in regulating inflammatory responses. Animal models have studied the role of TLR8 in allergic disease; however, translation of those studies into human biology is difficult due to the realisation that TLR8 function in mice differs from human TLR8. In humans, TLR8 and TLR7 are expressed in separate cell populations, but in mice, both are expressed together, and TLR8 is involved in regulating TLR7 (Paul et al. 2016; Tran, Manzin-Lorenzi & Santiago-Raber 2015).

The indication for atopy-independent TLR7 deficiency is surprising considering the body of evidence suggesting a role in counterregulating type 2 responses. Gill et al. (2010) found a counterregulatory relationship between TLR7 and the IgE receptor, FcεRI, in pDCs. The finding that pDC express both TLR7 and FcεRI in abundance is intriguing as TLR7 induces Th1 immunity via IFNα production and FcεRI engagement inhibits IFNα production and induces Th2 immunity, most likely via CCL28 (Grayson et al. 2007). These findings indicate that pDCs have a role in regulating the balance between Th1 and Th2 responses and that this may be mediated via TLR7. FcεRI expression in allergic asthma patients inversely

correlated with IFNα response to influenza virus activated pDCs, and in addition, activation of FcεRI by IgE crosslinking further reduced the IFNα response and TLR7 gene expression (Gill et al. 2010). Conversely, TLR7 agonist or influenza virus stimulation reduced FcεRI receptor expression, indicating a counterregulation between the receptors (Gill et al. 2010). Because high blood IgE levels usually characterise allergic asthma, these results further support low TLR7 gene expression in allergic asthma and consequent IFN response impairment. On the other hand, these results suggest that TLR7 activation will downregulate FcεRI mediated immune response and suppress the Th2 skewed immune response that is characteristic of asthma and results in the symptoms. Others have supported the counterregulation between type I IFN production and IgE activation in pDC (Durrani et al. 2012; Schroeder et al. 2005).

Animal studies indicate that TLR7 agonists are capable of reducing Th2 cytokines in mice, though there is no consensus on whether they can induce Th1 cytokines. In several animal models, TLR7 agonists have been shown to reverse experimental asthma and allergies (Aumeunier et al. 2010; Du, Q et al. 2009; Moisan et al. 2006), as well as normalise asthma cytokine and remodelling (Camateros et al. 2007; Duechs et al. 2011) and associated gene expression (Camateros et al. 2009). It was shown that the TLR7 agonists alleviate acute asthma symptoms by reducing bronchoconstriction (Ekman, Adner & Cardell 2011; Kaufman, Fryer & Jacoby 2011). Studies have found that TLR7's ability to diminish asthma symptoms is variously mediated via IL10 and IFNγ (Nencini et al. 2015), regulatory T-cells (Pham Van et al. 2011) or type I IFN (Matsui et al. 2012) depending on the ligand used. Independently of the Th2 counteracting abilities, a TLR7 agonist was also shown to directly reduce IgE generation in B cells, adding another means for reducing allergies (Shen, Lu & Wu 2008). Also, TLR8 agonist was shown to be a potent inhibitor of an allergic response in dogs (Royer et al. 2016).

Animal models and ex vivo human studies (Edwards, S et al. 2013) suggest that TLR7/8 activation has the potential to reduce the allergic and asthma inflammation and TLR7/8 agonists are therefore highly promising for drug development purposes. The TLR7/8 agonists in current use are conventionally used topically against external anogenital warts and basal cell carcinoma. Since it was first discovered that they could skew the CD4+ Th0 cytokine response towards Th1 (Wagner et al. 1999), their usefulness in allergic disease has been investigated. Also, the activation of TLR7 and TLR8 has been studied as vaccine

adjuvants as they seem to be able to activate immunity effectively (Rajagopal et al. 2010; Siebeneicher et al. 2014). Some TLR7 agonists developed and trialled for reducing asthma, and allergic rhinitis symptoms have progressed to phase 2 (Anwar et al. 2019; Leaker et al. 2012), and a TLR8 agonist has shown potential as an allergic rhinitis modulator due to its Th1 polarising cytokine repertoire (Horak 2011).

In addition to the Th2 modulating effect, TLR7 agonists relax human airway smooth muscle strips in vitro (Drake et al. 2013) previously modelled only in animals (Drake et al. 2013; Ekman, Adner & Cardell 2011; Kaufman, Fryer & Jacoby 2011). Interestingly, the mechanism of bronchodilation may be independent of TLR7 signalling and instead signify an off-target effect of the quinoline containing TLR7 agonists (Larsson et al. 2016), further supporting the development of TLR7/8 agonists as asthma medications.

### 1.4.4. Asthma genes associated with immune function

The notion that asthma has a strong genetic basis is well established, with twin studies suggesting that genetics explain 36–77% of the asthma risk (March, Sleiman & Hakonarson 2013). Asthma is thought to be a complex polygenic disease, so large population-level studies such as Genome-Wide Association Studies (GWAS) are needed to find associated genetic variants that may individually explain only a small amount of the risk of asthma. The 17q12–21 locus is important in childhood-onset asthma and includes the ORM1-like protein 3 (ORMDL3) and Gasdermin-B (GSDMB) genes, although it is notable that no association with allergy risk was found within this locus (Dijk et al. 2013). Instead, the *IL6R* gene, which is the receptor for IL6, is significantly associated with atopic asthma (Ferreira et al. 2011), and importantly, IL6 was found elevated in asthma and associated with severe asthma (Wood et al. 2012). In other studies, *IL18R1, IL33, SMAD3, HLA-DQ* and *IL2RB* loci have been associated with asthma (Zhang, Y, Moffatt & Cookson 2012). Many of these associated genes are related to antigen presentation, cytokine signalling and epithelial repair, suggesting that people with asthma have inappropriate immune responses and abnormal repair mechanisms, though there is little evidence in GWAS that genetic variants in type I IFN genes or IFNAR are associated with asthma risk. There may be a need for genetic studies that specifically focus on people with asthma who experience very high rates of respiratory viral infections.

A very recent GWAS study that included over 400, 000 people found an association between childhood asthma and the SNP rs850637 on X chromosome, in the vicinity of both *TLR7* and *TLR8* genes (Ferreira et al. 2019). The SNP was further confirmed in a validation cohort and presents a novel finding as most asthma GWAS published to date have not analysed the X-chromosome. Some candidate-gene studies have reported an association between variants in the TLR7/TLR8 region and asthma (Genov et al. 2014; Moller-Larsen et al. 2008; Nilsson et al. 2012; Törmänen et al. 2017), but these were small studies with relatively weak associations.

Another study searched for associations between SNPs in genes involved in antiviral immunity, asthma severity and respiratory infections in children (Loisel et al. 2016). The genes that appeared most significantly associated – *STAT4* and *MX1* – have been replicated in two other cohorts. Other key genes identified were *JAK2*, *VDR*, *DDX58*, and *EIF2AK2*. Many of those genes are involved in Th1 and Th2 T cell differentiation or IFN pathways. This study provides evidence that functional polymorphisms increase risk of asthma and respiratory infection severity in children.

### 1.4.5. Sex-dependent immune responses

Given that *TLR7* and *TLR8* are located on the X chromosome, it is relevant to review the epidemiological studies examining associations between sex and asthma risk. Pre-pubescent boys are more likely to have asthma than girls (Zein & Erzurum 2015), whereas their asthma symptoms often diminish in adulthood. The opposite is true for girls: lower prevalence in childhood is balanced by higher rates of asthma in adult women. The National Health Survey reveals a 'cross-over' at the age of 20–24 when there is similar prevalence in both genders (Clarke, Norris & Schiller 2017). The timing of the crossover suggests a strong hormonal association with asthma risk, and higher testosterone levels are known to be protective against type 2 immunity, in men (Laffont et al. 2017).

In relation to TLR7/8, Berghofer et al. (2006) found that TLR7 induces more IFNα in pDCs in females than in males. This finding is significant, considering the gender bias in asthma and the known associations between asthma and TLR7/8. Bias in TLR7/8 function is also significant for autoinflammatory diseases such as systemic lupus erythematosus SLE, in which there is high type I IFN production, and which is nine times more prevalent in females than males (Krieg 2007). Another study found there is a difference between the sex and age

and the adaptive immune response to RV, pre-menopausal women being the most efficient responders (Carroll et al. 2010). This result supports Berghofer's findings that the TLR7 mediated sex bias leads to more effective IFNα production women but also indicates hormonal influence. Laffont et al. (2014) expanded on the work of Berghofer in establishing sex differences in TLR7-mediated IFNα production. They used a humanised mouse model with human cord blood cells from men and women to show how the immune system works differently in female and male hormonal environment. They showed that the female environment and genetic factors independently influence IFNα production.

The sexual dimorphism in the immune response appears to be common to many stimuli and immune conditions, as reviewed by Furman (2015). Men show a weaker response to viruses and are more susceptible to viral infections than women, which relates to reduced cytokine production. This phenomenon has been shown across viral diseases such as HIV and HPV as well as in weaker response to vaccines. As a consequence, there has been discussion whether men and women should be treated with different vaccine doses, and indeed, retrospective analysis of previous studies reveals that vaccine trials deemed unsuccessful were, in fact, successful – in women. In addition to the work of Laffont et al. (2014), several other studies imply that oestrogen enhances the immune response while androgen dampens it (Kadel & Kovats 2018). However, the enhanced immune responses may come with a downside, as females are at more risk of mortality from influenza infections as they tend to respond with a "cytokine storm" leading to worsened symptoms in cell level (Robinson, DP et al. 2011). These findings reiterate the importance of considering sex-associated differences when studying antiviral and asthma immune responses.

## 1.5. Summary

The healthy immune defence against the common cold virus RV involves TLR7/8-mediated type I IFN and type 1-biased cytokine production. T-helper cell polarisation towards Type 1 response, and possibly ILC1 cells, seem to be a necessary part of the healthy antiviral immune response. Asthma patients have been reported to suffer from worse RV infections that have been associated with increased viral loads and imbalance between type 1 and type 2 immunity. Investigations into deficiencies have revealed deficiencies in type I and type III IFN production and TLR7/8 function, which may mediate this imbalance. The literature is controversial, and in disagreement with the asthma phenotypes that are associated with the antiviral immune response deficiencies, however many of the studies examined only small study groups. Genetic variation in TLR7/8 has been associated with asthma and may influence the deficient TLR7/8 reported in asthma. Strong evidence indicates that immune responses may vary with sex, further complicating the issue.

## 1.6. The objectives and aims of this research

Three primary aims of this study are:

1. To determine what clinical and immunological parameters are associated with self-reported respiratory infection frequency, the presence of asthma, asthma severity and control, with a specific focus on the role of *TLR7/8* gene expression and function, and the extent to which this differs between women and men.

2. To examine what host and transcriptional variations predict variation in the potency of antiviral type I IFN responses *in vitro* and determine how transcriptome patterns vary between individuals with high and low antiviral type I IFN responses.

3. To examine the functional impact that genetic variations in the *TLR7/*8 region has on TLR7/8 gene expression, downstream cytokine production, antiviral immunity, and immune cell counts in peripheral blood.

# Chapter 2: CLEC4C gene expression can be used to quantify circulating plasmacytoid dendritic cells

## Overview

Increasing sample size for a research study decreases the chance of type I and II errors tremendously, however, increased sample processing time, and complexity poses a considerable burden for study completion. To complete the aim of 300 recruits for the current study, we were required to limit sample processing time to avoid considerable delays to proposed timelines. Plasmacytoid dendritic cells (pDC) are of considerable interest to the current study because they are the main producers of type I interferon, express TLR7, a receptor of interest for this project, and are recognised to play a role in both asthma and antiviral immunity. Quantifying pDC in each sample by the standard method of flow cytometry was not considered practical as that would have increased sample processing time and cost. However, because quantifying cells by flow cytometry is based on unique receptor detection on each cell type, we hypothesised that gene expression of these same unique receptors utilised in flow cytometry could be used to quantify pDC in a sample. In this published study, we identify two receptors (CLEC4C and LILRA4) unique to pDC, measure their gene expression with RT-PCR, and compare the results obtained to CLEC4C and LILRA4 frequency measured by flow cytometry in peripheral mononuclear cells and whole blood. In this published chapter, we show that the two measurements are moderately correlated, justifying the use of *CLEC4C* gene expression as a tool to estimate pDC quantity in circumstances where flow cytometry is not practical. These measurements were employed for the subsequent chapters in this thesis. We show herein that *CLEC4C* gene expression is a robust indicator of pDC quantity that is unaffected by cell activation, whereas, the other evaluated gene, *LILRA4,* was deemed unreliable as a cell marker in activated cells.

*The following published article has been edited for in-text references.*

# CLEC4C gene expression can be used to quantify circulating plasmacytoid dendritic cells

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Declarations of interest:

Authors have no conflicts of interest to disclose.

Keywords:

pDC; CLEC4C; CD303; BDCA2; LILRA4; ILT7; CD85g; RT-PCR; FACS; gene expression; transcriptome; PBMC; whole blood

## Abstract

Plasmacytoid dendritic cells (pDC) are an important type I interferon producer that play an important role in the first line of host defence during viral infection. Abnormalities in pDC numbers and function have been associated with several health conditions. Quantifying pDC is important for understanding pDC related immune responses in viral infections and other diseases, however the current methods for quantifying pDC using flow cytometry have limited utility in large cohort studies involving multiple centres with limited access to flow cytometry. We reasoned that examining gene expression of the pDC marker C-type lectin domain family 4 member C (CLEC4C, also known as CD303 and BDCA2) in combination with pDC exclusive leukocyte immunoglobulin like receptor A4 (LILRA4, also known as CD85g and ILT7) might provide a more practical method that could be applied to multi-centre studies. Our results show a moderate correlation between pDC numbers measured by surface staining and *CLEC4C* gene expression in whole blood (rho=0.39, P=0.037, as well as a high correlation between *CLEC4C* gene expression in whole blood and peripheral blood mononuclear cells (rho=0.79, P<0.001). *LILRA4* gene expression did not provide additional useful information. Our results indicate that measuring *CLEC4C* gene expression can provide an alternative method for quantifying pDC numbers in human samples.

## Highlights

- *CLEC4C* gene expression measurement can be used to quantify relative pDC count.
- Gene expression correlates moderately with pDC surface staining.
- This method enables estimation of pDC counts retrospectively from transcriptome data

## 2.1. Introduction

Plasmacytoid dendritic cells (pDC) are recognised as the predominant type I interferon producing cell type in the primary antiviral response. Not only do pDC constitutively express viral nucleic acid-sensing pattern recognition receptors such as toll-like receptors, pDC contribute well over 90% of the type I interferon produced during a viral infection (Xi et al. 2015), which makes them highly relevant to host defence and pathogenesis in a variety of specific diseases. Alterations in numbers and function of pDC have been associated with delayed viral clearance (Dhamanage, Thakar & Paranjape 2016), autoimmunity, allergic disease and cancer (Swiecki, Melissa & Colonna 2015).

While there are a number of methods to study pDC function in experimental animals, studying pDC in human diseases presents several challenges, given that they are a rare leukocyte population in peripheral blood, accounting for <0.4 % of peripheral blood mononuclear cells (PBMC). Both pDC purification and pDC depletion (Xi et al. 2017) with immuno-magnetic beads or fluorescence activated cell sorting (FACS) (Kassianos et al. 2010) have been used to study pDC function. Numbers of circulating pDC can be measured accurately with flow cytometry, but this usually requires fresh blood samples which is not practical in large multi-centre cohort studies where there may not be ready access to flow cytometry. Recently, small volume blood samples have been used to analyse the transcriptome, proteome and metabolome (Urrutia et al. 2016) in various human diseases. With the advent of methods to collect and stabilise blood samples for subsequent gene expression analysis, there is now a convenient and accessible tool which can be used in large scale research studies, especially where there is limited capacity for sample processing in the field. We therefore reasoned that measuring the expression of pDC specific genes might provide a more convenient method for researchers to estimate pDC numbers in blood samples, especially in situations where flow cytometry is not readily available.

CLEC4C and LILRA4 are cell surface proteins that are expressed exclusively on pDC (Cao & Bover 2010; Xi et al. 2017). CLEC4C is a C-type lectin domain family 4 member C (also known as CD303 and BDCA-2) known to regulate antigen presentation and type I interferon production (Dzionek et al. 2001). Leukocyte immunoglobulin like receptor A4 (LILRA4; also known as ILT7 and CD85g) regulates TLR-mediated type I interferon production in pDC (Cao & Bover 2010) providing an important negative feedback loop for type I interferon

production. We hypothesised that the relative gene expression of *CLEC4C* and *LILRA4* measured by real time PCR would correlate with pDC cell numbers ($CLEC4C^{+}LILRA4^{+}CD14^{-}$ cells) measured by flow cytometry. Our findings show a moderate correlation score of 0.39 (P=0.037) between *CLEC4C* gene expression and pDC cell numbers measured with flow cytometry, thus providing an alternative method for estimating the pDC numbers in circulating blood.

## 2.2. Methods

### 2.2.1. Participants

We recruited 29 participants, of which 19 were female, with a mean age of 38.6 ± 13 years. All participants were healthy without respiratory infections within 4 weeks prior to their study visit. Metro South Health Human Research Ethics Committee approved the study, and all participants provided written consent.

### 2.2.2. Sample preparation

10 ml of heparinised blood and a PAXgene blood tube were collected from each participant. An aliquot of 1ml of heparinised blood was used to evaluate the percentage of the pDC in the whole blood using flow cytometry (section 2.2.3). PBMC were isolated from the remaining blood by density gradient centrifugation using Lymphoprep (Stemcell technologies, Vancouver, Canada) as described previously in Roponen et al. (2010). 1 x $10^6$ cells were used to evaluate the percentage of pDC in PBMC (section 2.2.4) and 4 x $10^6$ PBMC were stored in RNAprotect (Qiagen, Hilden, Germany) for RNA extraction and subsequently RT-PCR (section 2.2.5).

### 2.2.3. Whole blood cell preparation

300ul of whole blood per sample was washed once with 1ml of FACs buffer (1% heat-inactivated foetal bovine serum in phosphate-buffered saline; foetal calf serum; Bovogen Biologicals, Keilor East, VIC, Australia). The red blood cells were then lysed with 2ml of 1X BD FACS lysing solution (BD FACS™ lysing solution, BD Cat#349202, San Jose CA, USA) for 30mins at dark and room temperature. After centrifuging, the supernatant was removed and cell pellet was washed with 1ml of FACs buffer followed by surface staining (section 2:382.2.4).

### 2.2.4. Surface staining and flow cytometry analysis

The prepared cells (section 2.2.3) and isolated PBMC (section 2.2.2) were washed with FACs buffer and then stained with CLEC4C-PE (CD303, Miltenyi Biotec Cat# 130–090–511, RRID: AB_244168), CD14-PerCP (BioLegend Cat# 301824, RRID: AB_893251) and LILRA4-APC (CD85g, Thermo Fisher Scientific Cat# 17–5179–41, RRID: AB_10597138) in FACs buffer for 30min at 4°C in dark. The cells were then washed twice with the FACs buffer

and fixed in 2% paraformaldehyde. A total of ~500,000 gated events were acquired using LSRFortessa X-20 (BD Biosciences, San Jose, CA). Data was analysed using the FlowJo Tree Star software (version 7.6.1; FlowJo LLC, Ashland, OR, USA).

### 2.2.5. RNA extraction and quantitative RT- PCR analysis

RNA was extracted from the PAXgene tube using Preanalytix blood RNA kit (Qiagen, Maryland, USA) or from the PBMC stored in RNAprotect using RNeasy mini kit (Qiagen, Maryland, USA) plus DNase II digestion protocol. RNA was reverse-transcribed to cDNA with sensiFAST cDNA synthesis kit (Bioline, London, UK). Relative quantitative RT- PCR was performed from the cDNA with PowerUp SYBR Green (Applied Biosystems, CA, USA) mastermix with Lightcycler 480 (Roche) machine. Custom primers were purchased from Geneworks (SA, Australia) with sequences shown in appendices. Delta Ct of *CLEC4C* and *LILRA4* gene expression was obtained against the mean Ct of the two reference genes *B2M* (Beta-2-Microglobulin) and *UBC* (Polyubiquitin-C precursor) (primer sequences in appendix Table 2:1).

### 2.2.6. Statistical analysis

Nonparametric (for FACS measurements) or parametric rank correlation test were performed between paired samples in R version 3.4.2 (R Core Team 2018) with cor.test() function. To compare paired samples from the same individuals, the Wilcoxon signed rank test was performed using GraphPad Prism 6 for Windows (GraphPad Software, San Diego, CA, USA). A two-sided $p$-value<0.05 was considered significant.

## 2.3. Results and discussion

To validate gene expression quantification as a measurement tool we compared relative quantification by RT-PCR to the current 'gold standard', namely surface staining with FACS. In theory, the pDC frequency in each of the measurements should correlate but sample processing and technical variability were expected to produce variations.

LILRA4 was previously identified as being stably expressed in unstimulated pDC compared to other immune cell types (Cho et al. 2008). CLEC4C was recognised as a pDC specific cell marker in 2001 (Dzionek et al. 2001), and we previously showed that it is markedly reduced in pDC depleted cell populations (Xi et al. 2017), therefore we used those two markers to detect pDC population (Figure 2:1:A). We compared whole blood to PBMC, and as expected the pDC frequency was higher in PBMC than in whole blood (Figure 2:1:B).

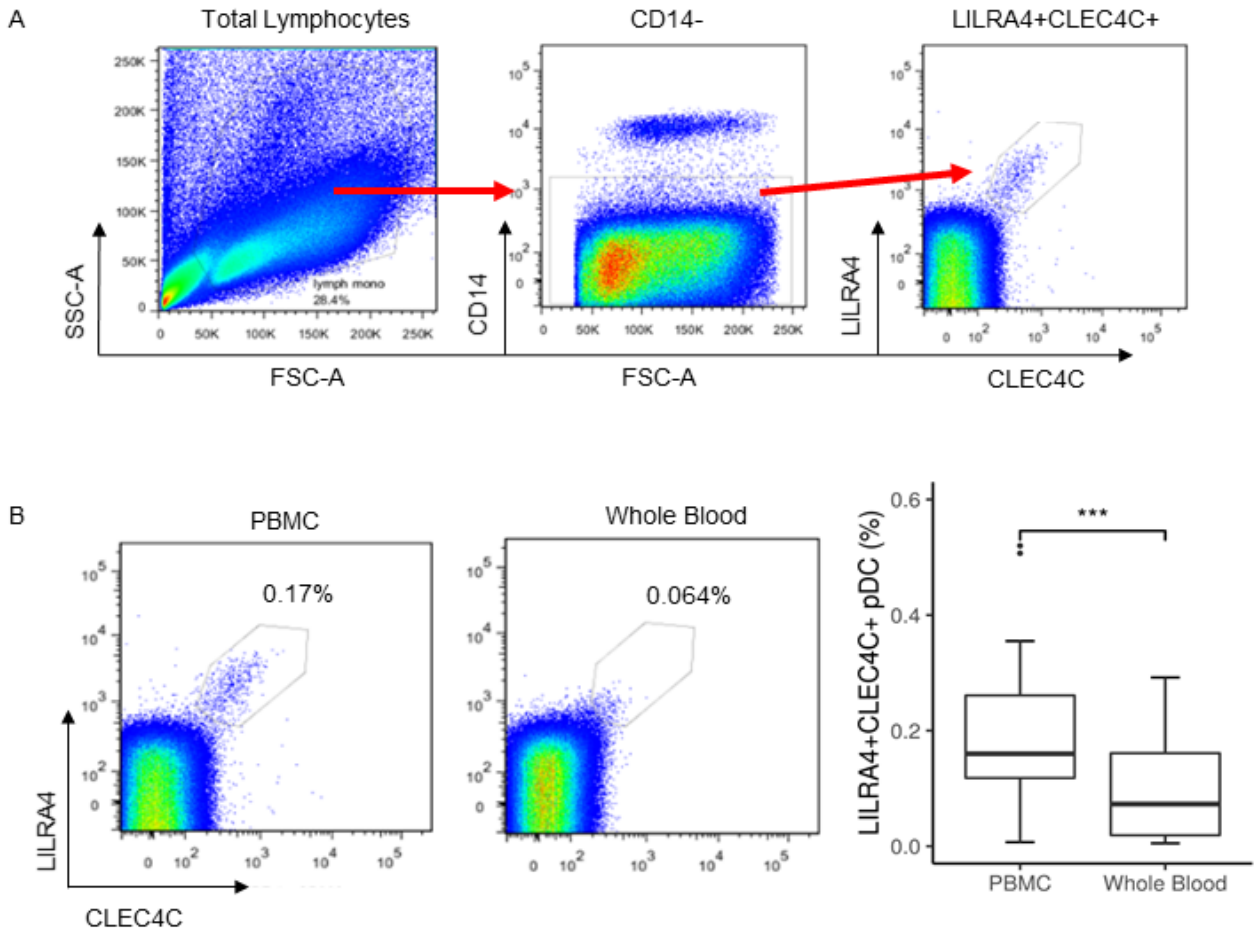


*Figure 2:1 Frequency of LILRA4 and CLEC4C expressing pDC in PBMC and whole blood. A) Top panels shows the gating strategy. Firstly, we gated on lymphocytes and monocytes based on forward and side scatter properties. CD14+ cells were then excluded, and the percentage of CLEC4C+ LILRA4+ cells was analysed. B) The percentage of*

*CLEC4C+LILRA4+ cells was enumerated in PBMC and whole blood. Note that LILRA4+CLEC4C+ pDC were more frequent in PBMC than in whole blood (Mann–Whitney U test, p-value 1.2e-07, n = 29).*

LILRA4 and CLEC4C expression were more strongly correlated in PBMC than in whole blood (Figure 2:2). In fact, the correlation coefficient for LILRA4 versus CLEC4C was similar with both the RT-PCR method (cor=0.84) and FACS method (rho=0.89) in PBMC. However, in whole blood, the correlation co-efficient for LILRA4 versus CLEC4C was higher for FACS (rho= 0.82) than for RT-PCR (cor = 0.7). It seems likely that the lower pDC frequency in whole blood, and the presence of additional cell types, increases the variability between samples and reduces the correlation coefficient.

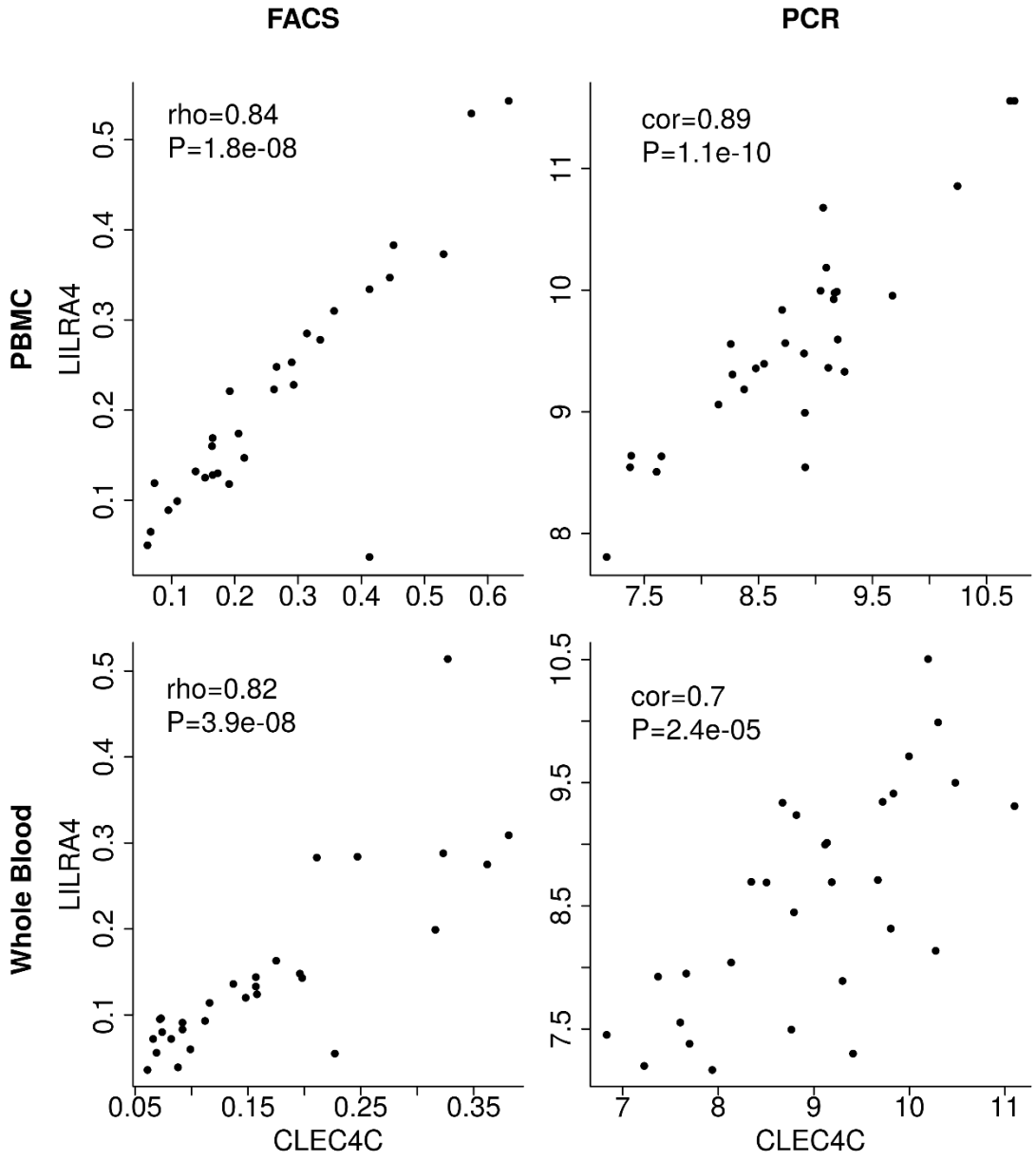


*Figure 2:2. Correlations between CLEC4C and LILRA4 as measured with FACS or with RT-PCR in PBMC or whole blood. Correlation coefficient is cor for Pearson correlation test performed for normally distributed PCR data and Rho for Spearman correlation test of nonparametric FACS data.*

Overall, correlation scores were high (Figure 2:2), so we reasoned that the combined use of the two markers might further increase the precision of the method. To compare the two methods for pDC measurement, we correlated the mean frequency of $CLEC4C^{+}LILRA4^{+}CD14^{-}$ cells against the mean *LILRA4* and *CLEC4C* gene expression for each sample in PBMC and whole blood. Correlation analysis (Figure 2:3:A) showed that both samples correlated highly for FACS measurements (rho=0.76, *p*=1.6e-06), and

similarly for RT-PCR samples (cor=0.7, *p*=2.6e-05). We concluded that both methods are accurate and that is likely to be relevant for studies with large sample sizes.

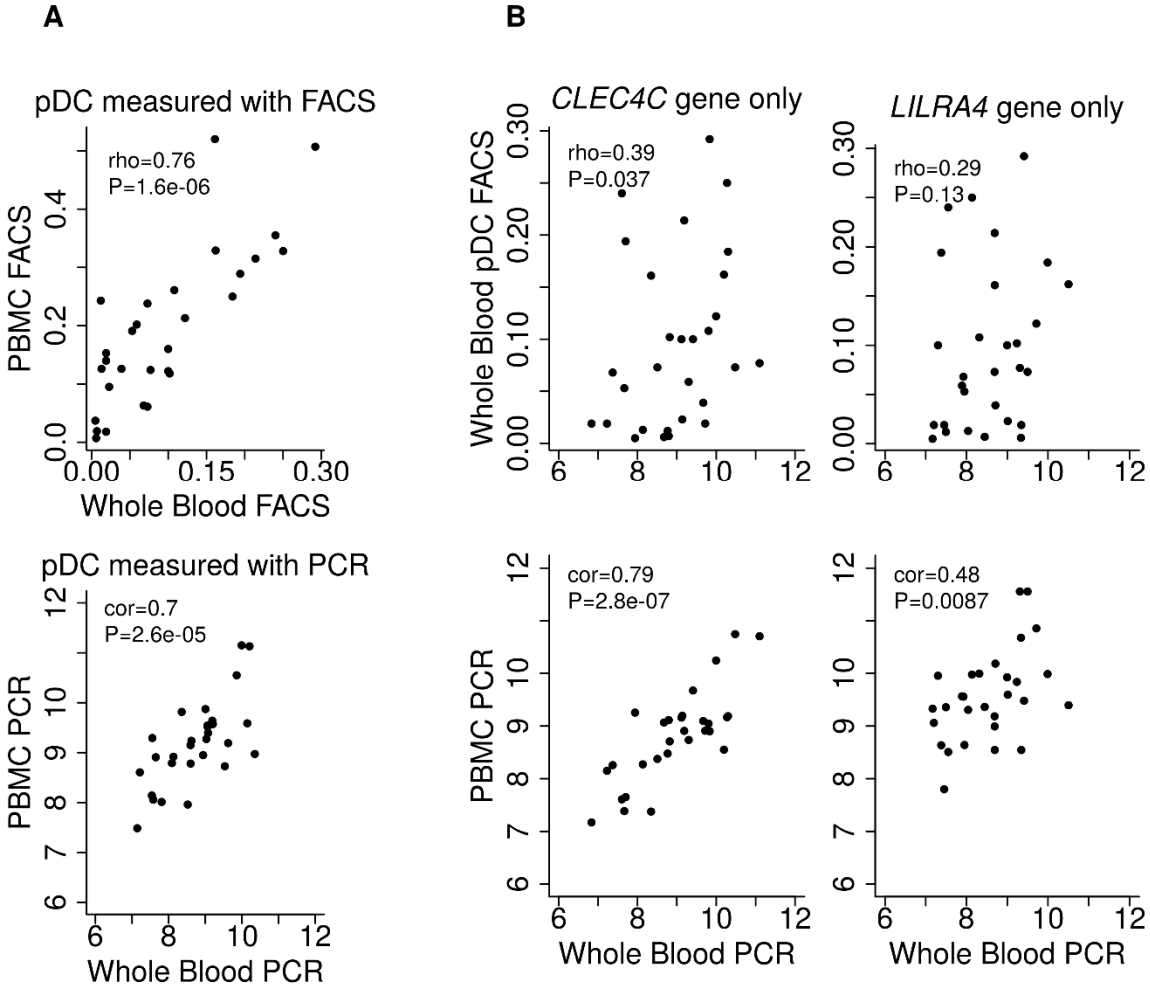


*Figure 2:3. Correlations between PBMC or whole blood with combined or single use of gene expression. A) Correlation between measures of pDC numbers in whole blood and PBMC with FACS above and PCR below. B: Correlation of pDC measurements using CLEC4C gene expression alone (left) or LILRA4 gene expression alone (right) between whole blood PCR and FACS above or PBMC PCR below. (rho = Spearman correlation coefficient, cor = Pearson correlation coefficient, P = P-value, sample size n = 29)*

However, we saw no significant correlation between mean RT-PCR and FACS measurements in PBMC (cor=-0.019, P=0.31) or whole blood samples (rho=0.35, P=0.067) to compare the relative pDC count of individuals. Since the whole blood sample correlation reached near significance, we then examined how individual gene expressions perform in whole blood. *CLEC4C* gene expression alone in whole blood samples correlated moderately with the mean frequency of $CLEC4C^{+}LILRA4^{+}CD14^{-}$ cells measured by FACS (Figure 2:3:B upper panel, rho=0.39, P=0.037). *LILRA4* gene expression was not significantly correlated

with pDC measured by FACS (rho=0.29, P=0.13). *CLEC4C* gene expression alone correlated strongly between PBMC and in whole blood samples (cor=0.72, P=2.8e-07, Figure 2:3:B lower panel)) – at similar level to that seen with FACS (rho=0.76, *p*=1.6e-06, Figure 2:3:A upper panel. These results indicate that using both pDC markers for gene expression analysis is not beneficial. *CLEC4C* appeared to be a more robust marker than *LILRA4*, as its use alone showed the best correlation between the two methods (FACS vs RT-PCR) and showed stronger correlation between the two samples types (whole blood vs PBMC).

Because of research interest in pDC biology during viral infection, it is important that selected pDC markers are unaffected by the state of the pDC activation. In our results, CLEC4C performed consistently across the two samples types and methods but LILRA4 did not, especially when using RT-PCR on whole blood samples. The available evidence suggests that *LILRA4* gene expression may be more influenced by pDC activation status than *CLEC4C* gene expression (Cho et al. 2008; Xi et al. 2017) thereby making *LILRA4* gene expression unstable as a marker for estimating pDC numbers. Therefore technical factors and/or biological variability (e.g. subtle changes in cell activation induced by sample processing) could explain why *CLEC4C* gene expression appears to provide a more reliable estimate of pDC numbers than *LILRA4* gene expression. Moreover, we recognise that a low participant number could have limited the study. For use with larger studies, the method requires optimisation and measurement of a known cDNA standard measurement across batches as an internal control.

In conclusion, while flow cytometry remains the ‘gold standard’ for quantifying blood pDC, analysis of *CLEC4C* gene expression does provide a valid method for assessing relative pDC numbers across samples. Assessing *LILRA4* gene expression does not provide additional useful information. Here we provide evidence that FACS measurements of pDC and RT-PCR measurements of *CLEC4C* gene expression correlate to a reasonable degree, so we recommend the use of CLEC4C and LILRA4 surface staining or *CLEC4C* gene expression analysis for estimating pDC numbers in human blood samples. Our findings are relevant to researchers with an interest in measuring pDC numbers in large patient cohorts, especially in field studies where flow cytometry is not readily available.

## 2.4. Appendices:

| Gene | Direction | Primer sequence 5' – 3' |
|---|---|---|
| *LILRA4* | forward | *CTCGTGGTCTCAGGAGCAACT* |
| | reverse | *CAGTCTTGGAATCTGACTTCTTTTGT* |
| *CLEC4C* | forward | *CCTCTGTCTGACCCTGCAT* |
| | reverse | *GCCAAGCCCTTAGATCCTT* |
| *UBC* | forward | *GCAGTTCTTGTTTGTGGATCGCT* |
| | reverse | *TGACATTCTCGATGGTGTCACTGG* |
| *B2M* | forward | *AGGCTATCCAGCGTACTCCAAAGA* |
| | reverse | *CGGATGGATGAAACCCAGACACAT* |

*Table 2:1. Relative quantity of CLEC4C and LILRA4 gene expression was determined using delta Ct of CLEC4C and LILRA4 gene expression against the mean Ct of two reference genes B2M and UBC.*

# Chapter 3: Clinical features and TLR7/8-mediated antiviral immunity impact respiratory infection frequency and asthma in a sex-dependent manner

## 3.1. Introduction

In this chapter, we examine the clinical and immunological parameters that are associated with respiratory infections and asthma.

Respiratory infections place asthma patients at an increased overall risk of experiencing exacerbations, especially severe exacerbations requiring hospitalisation (Busse, Lemanske Jr & Gern 2010; Johnston et al. 1995). In addition to increased asthma symptoms, evidence shows that rhinovirus (RV) infections are more severe in asthma patients, resulting in a lower respiratory tract infection more often than in their healthy counterparts (Corne et al. 2002). More severe infections in mild asthmatics were also shown with an experimental RV infection (Message et al. 2008). A recent study of over 100,000 participants revealed that asthma increased the risk of being hospitalised for infections in general (Helby et al. 2017), indicating increased susceptibility to infections and their consequences. Despite the current evidence for increased severity of respiratory infections in asthma, it is unclear whether asthma patients are at a greater risk of contracting respiratory infections more frequently than healthy people. Ramette et al. (2018) showed that asthma is associated with frequent and prolonged infections in children at one, four and six years of age, but for adults, the frequency of confirmed RV infections is similar in asthma patients compared with healthy partners (Corne et al. 2002).

The severe course of respiratory infections experienced by asthma patients point to deficiencies in their antiviral immune responses. The aberrant immune response against viral infections is thought to be the result of asthma-related inflammation, but also inherited immune deficiencies could result in higher viral loads since many genes that have been associated with asthma risk are part of the immune response (Vicente, Revez & Ferreira 2017). A growing body of literature suggests that the production of type I interferon (IFN), a main antiviral cytokine, is deficient in a subgroup of people with asthma (discussed in section 1.4.2). Lower levels of type I IFN in people with asthma has been associated with specific features of asthma including eosinophilic airway inflammation and severe asthma symptoms (Baraldo et al. 2012; Sykes et al. 2014).

Type I IFN production is triggered by pathogen pattern receptors (reviewed in section 1.2.2). One of those is TLR7 that is activated by single-stranded RNA (ssRNA), the most abundant nucleic acid amongst respiratory viruses. TLR7 and its pathway molecules are essential for

sufficient type I IFN production, and deficiencies have been shown in response to RV in asthma patients (Pritchard et al. 2014). In addition to TLR7 function, IFNα production is affected by several other factors: fluctuation in plasmacytoid dendritic cells (pDC), the main interferon alpha (IFNα) producing cell type (Xi et al. 2015), sex and age (Carroll et al. 2010), and TLR8, another ssRNA receptor (Wang, J et al. 2006). All these complex factors may contribute to variations in IFNα production.

IFNα induced by TLR7 in pDC, as well as the proinflammatory cytokines induced by TLR8, are central in driving type 1-dominant antiviral immune responses. The type 2-oriented immunity in asthma antagonises type 1-responses, and there is evidence that TLR7 activation helps reduce type 2 immunity in asthma (Duechs et al. 2011). However, it is unclear if TLR7 levels are reduced in asthma and if it is associated with asthma severity, control or atopic asthma.

Deficiencies in the pDC-TLR7-IFN axis have been associated with reduced antiviral immune responses in asthma (Edwards et al. 2017; Lynch et al. 2014; Pritchard et al. 2014), but it is uncertain if this causes asthma patients to become susceptible to respiratory infections. Therefore, this study sought to determine what clinical and immunological parameters are associated with self-reported respiratory infection frequency, the presence of asthma, asthma severity and control, with a specific focus on the role of TLR7/8 gene expression and function. Further, we sought to determine to what extent these associations differ between women and men.

To address these questions, we compared clinical and immunological parameters in 150 asthma patients and 151 controls. We used multivariable linear regression model analyses to examine the associations between self-reported respiratory infection frequency and clinical and immunological parameters, including *TLR7/8* gene expression at baseline, TLR7/8 induced cytokine production, sex, age, body mass index (BMI), and circulatory immune cells, including pDC. We also examined the associations of these parameters with asthma, asthma severity and control.

Enhanced knowledge of deficiencies in antiviral immunity in asthma is essential to identify which patients are at an increased infection risk. Secondly, understanding deficiencies at the cellular level could eventually lead to the development of medication alleviating respiratory infection related asthma symptoms.

## 3.2. Materials and methods

### 3.2.1. Participants

#### *Participant selection and recruitment*

An asthma case-control study was set up to recruit 300 participants. The study received ethical clearance from the University of Queensland (project: 2008000037) and Metro South Human Research Ethics Committees (Reference: HREC/07/QPAH/146). We recruited participants from the Princess Alexandra (PA) Hospital campus and respiratory outpatient clinics, the University of Queensland campus, and through social media. All participants were informed about the study in layman wording and provided written informed consent before attendance.

Inclusion criteria were: ≥ 18 years of age, smoke exposure < 20 pack-years of cigarettes, full two weeks symptom-free from respiratory infections prior to the appointment and no sibling already included in the study. Asthma cases were required to have a current asthma diagnosis, have experienced asthma-related symptoms in the past year and be treated with asthma medication. Participants were clear of oral corticosteroid treatment for four weeks and inhaled corticosteroid medication for 24 hours before sample collection. Control participants must never have had an asthma diagnosis. Case and control groups were matched for sex and age, and equal proportions of participants less than 50 years of age and greater than 50 years of age were included, as this is the average age for menopause in Australian women. The participants reported their ethnic ancestry that is further discussed in Chapter 5 methods.

#### *Participant interview*

Each participant with asthma was assigned to a GINA (Global Initiative for Asthma) treatment step based on their medications, and this provided an indirect measure of asthma severity (https://ginasthma.org/). Participants also completed an ACQ6 (Asthma Control Questionnaire), which is a standardised diagnostic tool for assessing control of asthma symptoms within the past week (Juniper et al. 2006). An ACQ6 score of below 1.0 is considered well-controlled asthma. Both cases and controls answered a questionnaire about their demographics, history of asthma and allergic disease in their families, and annual respiratory infection frequency. Self-reported respiratory infection (cold) frequency per year

was assessed based on the following question: “How frequently have you had cold symptoms (at least one of these: fever, chills, sore throat, runny nose or cough for at least one day) per year?”

### 3.2.2. Data storage

Participant information was made anonymous by assigning a study identity number. Both participant and asthma questionnaires (see appendix) were generated using the Checkbox program (Watertown, USA) online, that participants could access to complete the questionnaires online before their appointment. The opted paper versions of the questionnaires are stored in a secure location, and the electronic copies of questionnaires are downloaded to a file and secured with a password in a restricted access server. Results from the sample measurements were recorded anonymously in a separate database.

### 3.2.3. Human rhinovirus 16

Human rhinovirus (RV)16 was grown in Ohio HeLa cells. Confluent cells were incubated with RV16 lysate at 37°C until 60% of the cells were lysed. The infected cells were disrupted with three cycles of freezing and thawing and subsequently clarified with a centrifugation step and Optiprep (Sigma Aldrich Pty Ltd, Germany) purification. Titration assay was then completed to determine the concentration of the virus.

### 3.2.4. Sample processing

We collected 41.5ml of peripheral blood that was used as follows: complete blood, differential leukocyte count and total IgE were measured by PA Hospital Pathology Department, PAXgene (PreAnalytiX, Hombrechtikon, Switzerland) tubes were collected for RNA extraction and incubated at room temperature for 2 hours, followed by 24 h at -20$^0$C and then stored at -80$^0$C, and heparinised blood samples were used for peripheral mononuclear cell (PBMC) isolation.

#### *PBMC and PMNC isolation*

PBMC were isolated with Lymphoprep (Stemcell technologies, Tullamarine Australia) density gradient centrifugation and washed twice before counting with RPMI medium 1640 (Lifetechnologies, Melbourne Australia) supplemented with 2% Foetal Calf Serum (FCS) and PSG (Lifetechnologies, Melbourne Australia, 100u/ml Penicillin, 100µg/ml Streptomycin

and 0.292mg/ml L-glutamine). The remaining granulocytes were purified with in-house red blood cell lysis buffer (0.144M $NH_4Cl$, 1mM $NaHCO_3$) and stored at -20°C.

***PBMC treatment***

Freshly isolated PBMC were rested for one hour before culturing in RPMI medium 1640 supplemented with 2% FCS and PSG for 24 hours with either MOI = 2.5 of RV16 or 1μM TLR8 specific agonist VTX-2337 (Sapphire Bioscience, Waterloo Australia), as determined by our dose-response optimisation and previous literature (Lu et al. 2012). The supernatant was stored at -20$^0$C and the PBMC cell pellets were stored in RNAprotect (Qiagen, Hilden, Germany) at -80$^0$C.

***Cytokine measurement***

Enzyme-linked immunosorbent assay (ELISA) of IFNα (pan-specific, Mabtech Ab, Sweden), IL12 [IL12 (p70), BD OptEIA, BD Biosciences, USA] and TNF (BD Biosciences, USA) were used to measure cytokine levels in PBMC supernatants. Cytokine ELISAs were controlled for technical and biological variability with biological duplicates.

***Gene expression quantification***

Gene expression of *TLR7*, *TLR8*, and *CLEC4C* from whole blood was measured as described in Chapter 2: section 2.2.5. The comparative Ct method (Schmittgen & Livak 2008) was applied using *B2M* (Beta-2-Microglobulin) and *UBC* (Polyubiquitin-C precursor) as reference genes. Custom primer sequences are listed in Table 3:1.

*Table 3:1 Primer sequences of the genes of interest and reference.*

| Gene | Direction | Primer sequence 5' – 3' |
|---|---|---|
| *CLEC4C* | forward | *CCTCTGTCTGACCCTGCAT* |
| | reverse | *GCCAAGCCCTTAGATCCTT* |
| *TLR7* | forward | *ATGGTGTTTCCAATGTGGAC* |
| | reverse | *GTTCGTGGGAATACCTCCAG* |
| *TLR8* | forward | *TCCTTCAGTCGTCAATGCTG* |
| | reverse | *CGTTTGGGGAACTTCCTGTA* |
| *UBC* | forward | *GCAGTTCTTGTTTGTGGATCGCT* |
| | reverse | *TGACATTCTCGATGGTGTCACTGG* |
| *B2M* | forward | *AGGCTATCCAGCGTACTCCAAAGA* |
| | reverse | *CGGATGGATGAAACCCAGACACAT* |

### 3.2.5. Statistical analysis

All statistical analyses were performed with R version 3.4.4 (R Core Team 2018). Variables were tested for normality and normalised using natural logarithm as required. Associations were tested with linear, logistic or ordinal logistic regression, as appropriate. Multivariable regression model included all antiviral immune variables of interest and potential clinical confounders to the dependent variable. Backward stepwise regression was used to fit the best multivariable model reduced to 3-4 most significant variables. The independent variables were. The difference in sample distribution was tested with a nonparametric Mann-Whitney *U* test as the distribution of all variables were skewed. Nonparametric Spearman's rank correlation test was used to test the correlation between observations. *p*-values < 0.05

were considered statistically significant. Chi-square test was used to test significant difference in distribution between groups.

## 3.3. Results

### 3.3.1. Participant demographics

We recruited 150 asthma cases and 151 control subjects. The characteristics of the two groups are summarised in Table 3:2. Most participants were of European descent, with smaller numbers of Asian descent. In comparison to controls, asthma cases comprised slightly more women and participants over 50 years of age, but these differences were not statistically significant (see Table 3:2). The asthma group reported more frequent colds ($p < 0.001$) and greater exposure to children ($p = 0.015$) than the control group. Moreover, the asthma group had significantly higher body mass index (BMI) (median BMI 27.00) than the control group (median BMI 23.00, $p < 0.001$). The asthma group had significantly higher numbers of total blood leukocytes, neutrophils, monocytes, eosinophils, basophils and platelets than the healthy group, suggesting a degree of systemic inflammation. As expected, total IgE levels were significantly higher in the asthma group than in the healthy group.

*Table 3:2 Characteristics of the study groups*

| | Asthma | Control | *p*-value |
|---|---|---|---|
| **n** | 150 | 151 | |
| **Women/Men (%)** | 97/53 (64.7/35.3) | 90/61 (59.6/40.4) | 0.431 |
| **Ancestry (%)** | | | **0.001** |
| **Unknown** | 1 (0.7) | 1 (0.7) | |
| **Africans** | 0 (0.0) | 1 (0.7) | |
| **Asians** | 6 (4.0) | 26 (17.2) | |
| **Europeans** | 133 (88.7) | 105 (69.5) | |
| **Mixed** | 10 (6.7) | 18 (11.9) | |
| **Cold frequency** | 3.00 [3.00, 5.00] | 2.00 [1.00, 3.00] | **<0.001** |
| **With children (%)** | 42 (28.2) | 24 (15.9) | **0.015** |
| **Age at donation (years)** | 35.00 [26.00, 47.00] | 32.00 [26.00, 44.00] | 0.309 |
| **Over 50 (%)** | 35 (23.3) | 31 (20.5) | 0.654 |
| **Smoked pack years** | 0.00 [0.00, 0.00] | 0.00 [0.00, 0.00] | 0.871 |
| **BMI ($kg/m^2$)** | 27.00 [23.00, 30.00] | 23.00 [22.00, 26.00] | **<0.001** |
| **Blood count** | | | |
| **White blood cells x $10^9$/L** | 6.50 [5.50, 7.70] | 5.90 [4.95, 6.80] | **<0.001** |
| **Platelets x $10^9$/L** | 269.00 [234.50, 301.00] | 240.00 [211.50, 271.50] | **<0.001** |
| **Neutrophils x $10^9$/L** | 3.68 [2.94, 4.57] | 3.21 [2.75, 4.03] | **0.003** |
| **Lymphocytes x $10^9$/L** | 1.92 [1.64, 2.43] | 1.87 [1.56, 2.20] | 0.072 |
| **Monocytes x $10^9$/L** | 0.53 [0.42, 0.63] | 0.46 [0.37, 0.57] | **0.003** |
| **Eosinophils x $10^9$/L** | 0.25 [0.15, 0.39] | 0.13 [0.08, 0.19] | **<0.001** |
| **Basophils x $10^9$/L** | 0.03 [0.02, 0.04] | 0.02 [0.01, 0.03] | **<0.001** |
| **IgE kU/L** | 143.00 [51.75, 346.75] | 34.00 [13.00, 100.00] | **<0.001** |

*n = study group size, with children = household with children under 15 years of age, over 50 = over 50 years of age, BMI = body mass index. Continuous variables are expressed as median [interquartile range] and categorical as frequency (percentage). p-value is shown for Mann-Whitney U-test between the two groups for continuous variables and Chi-square test for the categorical variables. Bolded p-values are statistically significant.*

### 3.3.2. Characteristics of the asthma group

Most asthma participants were either in GINA step 4 (n = 55, 37%) or GINA step 1 (n = 51, 35%) treatment groups. This likely reflects the recruitment of more severe patients from the clinic and mild asthma patients from the community. A relatively smaller number of participants were in GINA steps 2, 3 and 5 (Table 3:3). The relationships between GINA treatment step and asthma control, as measured by the ACQ6 score, are shown in Figure 3:1. Predictably, higher ACQ6 scores were seen in GINA step 5 participants. The overall median ACQ6 score was 0.50 [interquartile range (IQR) 0.21, 1.33], indicating good overall asthma symptom control.

Measures of age, BMI, ACQ6 score, having missed work in the past 12 months, hospitalisation for asthma and intake of oral corticosteroid medication were significantly different between the GINA step groups and followed an increasing trend from GINA step 1 through steps 3, 4 and 5 (Table 3:3), however the smallest GINA step 2 group deviated from those trends. Post-hoc tests with multiple testing correction indicated that the GINA step 5 participants were significantly older, had a higher ACQ6 score and were more likely to have taken oral corticosteroids in the past 12 months than participants in GINA step 1. Notably, self-reported cold frequency did not vary across the GINA step groups.

We also tested which variables contributed to the increase in ACQ6 score, *i.e.* asthma symptom control. Age, smoking and BMI were positively associated with ACQ6 score [ACQ6 increased 0.01 per 1 year ($p$ = 0.005), 0.03 per 1 smoked pack year ($p$ = 0.031) and 0.04 per 1 BMI unit increase ($p$ = 0.002)]. Having been hospitalised (ever, or in the past 12 months), having missed work or taken oral corticosteroid medication (in the past 12 months) were all associated with an increase in ACQ6 score [ACQ6 score increase of 0.44, 0.66, 0.39 and 0.69 ($p$ = 0.004, 0.11, 0.15 and $p$ < 0.001), respectively]. Self-reported cold frequency was not associated with ACQ6 score.

Although asthma severity and asthma symptom control were both influenced by common variables, blood eosinophil counts were similar across the GINA step groups (Table 3:3). However, eosinophil counts were positively associated with ACQ6 score (0.75 increase in ACQ6 score per 1 unit increase in eosinophilic count; $p$-value = 0.020). Blood neutrophil count and total IgE were not associated with ACQ6 score. Also, a history of having ever been hospitalised for asthma did not differ between asthma severity groups, whereas an increase in ACQ6 score was associated with both a history of asthma-related hospitalisation and hospitalisation in the past 12 months.

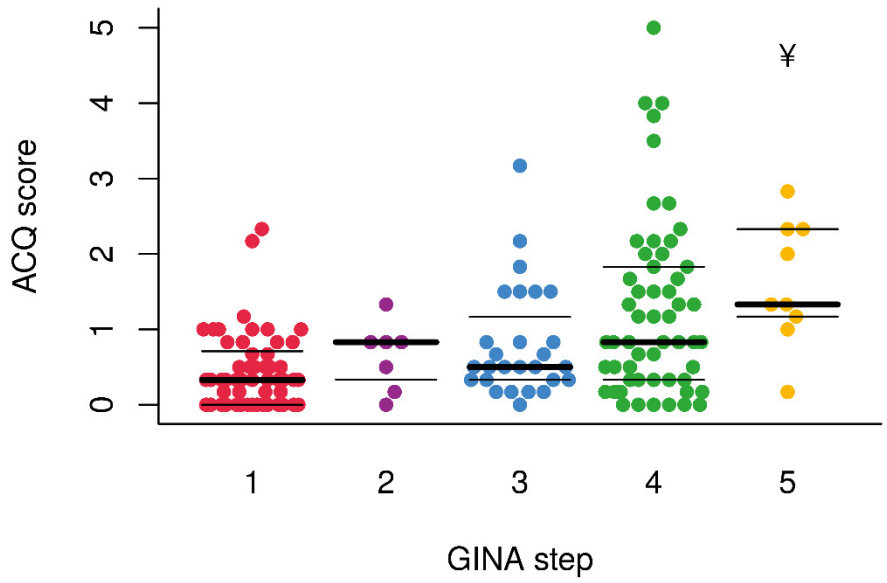


*Figure 3:1 ACQ6 score by GINA step.*
*ACQ6 score distribution by GINA step. The thick vertical line represents the median ACQ6 score, and the thin lines represent the interquartile ranges. ¥ designates a significant Mann-Whitney U-test with p-value < 0.0125 vs GINA step 1.*

Table 3:3 Asthma participants described by GINA step group and ACQ score

| | GINA step | | | | | | ACQ6 score | |
|---|---|---|---|---|---|---|---|---|
| | 1 Mild | 2 | 3 | 4 | 5 Severe | *p* (1 | Coef | *p* (2 |
| **n** | 52 | 7 | 27 | 55 | 9 | | | |
| **Women – n(%)** | 29 (55.8) | 7 (100.0) | 21 (77.8) | 38 (69.1) | 2 (22.2) | **0.004** | 0.04 | 0.823 |
| **Age at donation (years)** | 29.0 [24.0, 39.3] | 51.0 [33.5, 56.0] | 31.0 [25.0, 45.5] | 37.0 [29.5, 49.5] | 51.0 [42.0, 61.0] ¥ | **0.001** | 0.01 | **0.005** |
| **Smoked pack years** | 0.0 [0.0, 0.0] | 0.0 [0.0, 0.0] | 0.0 [0.0, 0.0] | 0.0 [0.0, 1.8] | 0.0 [0.0, 0.0] | 0.060 | 0.03 | **0.031** |
| **BMI (kg/m$^2$)** | 23.0 [21.0, 27.2] | 30.0 [26.5, 35.0] | 27.0 [22.5, 29.5] | 28.0 [24.5, 30.0] | 29.0 [27.0, 35.0] | **0.001** | 0.04 | **0.002** |
| **Cold frequency** | 3.0 [3.0, 5.0] | 3.0 [3.0, 12.0] | 3.0 [3.0, 5.0] | 3.0 [3.0, 5.0] | 5.5 [3.5, 8.2] | 0.658 | 0.01 | 0.557 |
| **ACQ6 score** | 0.3 [0.0, 0.7] | 0.8 [0.3, 0.8] | 0.5 [0.3, 1.2] | 0.8 [0.3, 1.8] | 1.3 [1.2, 2.3] ¥ | **<0.001** | | |
| **IgE kU/L** | 104.0 [48.2, 317.2] | 91.0 [49.5, 269.0] | 165.0 [66.0, 517.0] | 164.0 [52.5, 358.0] | 217.0 [31.0, 224.0] | 0.779 | 0.0001 | 0.282 |
| **Eosinophils x 10$^9$/L** | 0.2 [0.1, 0.4] | 0.2 [0.2, 0.5] | 0.3 [0.2, 0.4] | 0.3 [0.2, 0.4] | 0.2 [0.1, 0.4] | 0.146 | 0.75 | **0.020** |
| **Neutrophil x 10$^9$/L** | 3.3 [2.7, 4.1] | 4.4 [4.0, 4.6] | 3.7 [3.0, 4.7] | 4.0 [3.3, 4.7] | 3.6 [2.6, 6.3] | 0.055 | 0.08 | 0.133 |
| **Asthma diagnosis age (years)** | 1.0 [1.0, 2.0] | 1.0 [1.0, 1.0] | 1.0 [1.0, 2.5] | 1.0 [1.0, 2.0] | 1.0 [1.0, 3.0] | 0.668 | 0.09 | 0.366 |
| **In 12 months missed work for asthma** | 8 (15.4) | 0 (0.0) | 11 (40.7) | 26 (47.3) | 4 (44.4) | **0.002** | 0.39 | **0.015** |
| **Ever hospitalised for asthma** | 27 (51.9) | 3 (42.9) | 12 (44.4) | 33 (60.0) | 8 (88.9) | 0.161 | 0.44 | **0.004** |
| **In 12 months hospitalised for asthma** | 1 (1.9) | 0 (0.0) | 3 (11.1) | 7 (12.7) | 3 (33.3) | **0.026** | 0.66 | **0.011** |
| **In 12 months taken OCS** | 8 (15.4) | 2 (28.6) | 9 (33.3) | 27 (49.1) | 9 (100.0) ¥ | **<0.001** | 0.69 | **< 0.001** |

*Categorical variables are expressed as frequency (percentage), and continuous variables are expressed as median [interquartile range] for each GINA step group. Variables are tested for an association with ACQ6 score with univariate linear regression. BMI = body mass index, n = study group size, BMI = body mass index, ACQ = asthma control questionnaire, mo. = months, OCS = oral corticosteroid medication, cold frequency = frequency of self-reported respiratory infections. p (1 = statistical significance of the chi-square test for categorical variables or Kruskal-Wallis test for continuous variables between the GINA step groups. p (2 = statistical significance of univariate test with ACQ6 score as an outcome variable. Coef = β-coefficient for association. ¥ = post-hoc test p < 0.05 / 4 tests = 0.0125 vs GINA step 1.*

### 3.3.3. Associations between respiratory infection frequency, clinical variables, and antiviral immunity

#### *Respiratory infection frequency correlates with baseline TLR7 gene expression*

We examined whether TLR7/8-related antiviral immunity is associated with self-reported respiratory infection frequency. We tested for a correlation between respiratory infection frequency and *TLR7/8* gene expression in whole blood cells directly after sample collection, and TLR7/8 function as measured by IFNα, TNF and IL12 cytokine production in PBMC after TLR7/8 stimulation. We also measured *CLEC4C* gene expression in the whole blood samples, which indicates the relative quantity of pDC as described in Chapter 2. Of these variables, only *TLR7* gene expression in whole blood was in weak inverse correlation with respiratory infection frequency with borderline significance (Spearman rho = -0.11, $p$ = 0.06, Figure 3:2).

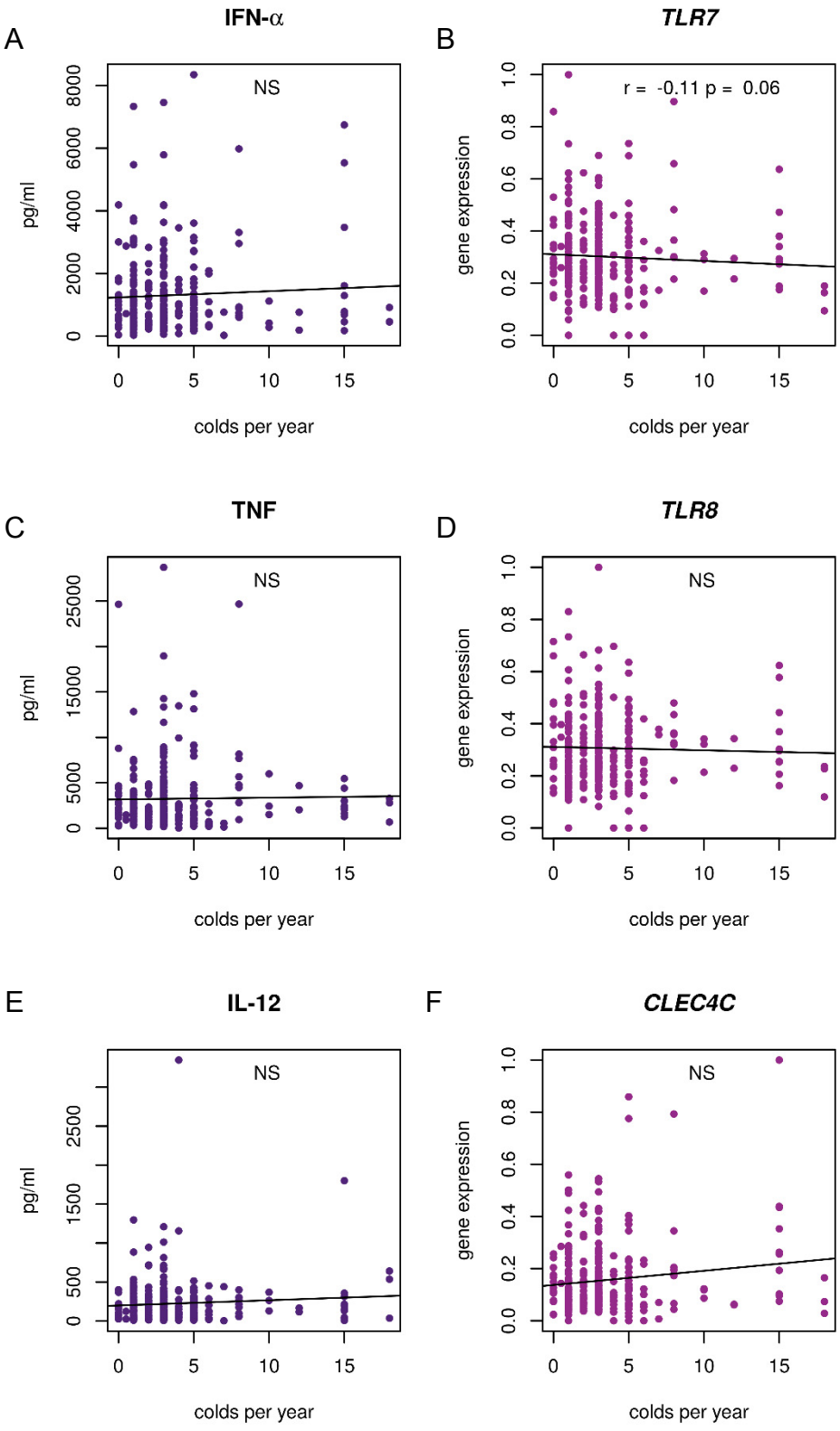


*Figure 3:2 Correlation between respiratory infection (cold) frequency and antiviral immune response variables.*
*Spearman rank correlation test was performed between cold frequency and cytokine (A) RV-induced IFNα, (C) TLR8 agonist-induced TNF and (E) IL12 production and gene*

*expression of (B) TLR7, (D) TLR8 and (F) CLEC4C in whole blood. NS = not significant, r = Spearman correlation score, p = p-value.*

### ***Determinants of respiratory infection frequency***

To mitigate the effect of potential confounders, we utilised a multivariable linear regression model to explain respiratory infection frequency with antiviral immune response variables. We included all the measured antiviral immune response variables in the linear model and controlled for exposure to young children, BMI, age and sex. Linear model fitting with stepwise reduction showed that the weak correlation between *TLR7* mRNA and cold frequency was no longer significant, but *CLEC4C* expression (used as an indirect measure of blood pDC numbers) was associated with self-reported cold frequency when adjusted for *TLR7* gene expression, age and BMI (Model 3:1).

*Model 3:1 Multivariable linear regression model for cold frequency*

| **Dependent variable: Cold frequency per year** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Univariate models** | | | | **Multivariable model** | | | |
| | **Est. Coef** | **CI 2.5%** | **CI 97.5%** | ***p*** | **Est. Coef** | **CI 2.5%** | **CI 97.5%** | ***p*** |
| ***CLEC4C* mRNA** | 0.46 | -0.058 | 0.98 | 0.081 | 0.63 | 0.09 | 1.16 | **0.02** |
| ***TLR7* mRNA** | -0.36 | -0.85 | 0.13 | 0.14 | -0.43 | -0.94 | 0.07 | 0.09 |
| ***TLR8* mRNA** | -0.22 | -0.73 | 0.30 | 0.41 | | | | |
| **IFNα pg/ml** | 2.0E-05 | -3.7E-05 | 7.7E-05 | 0.49 | | | | |
| **TNF pg/ml** | 4.3E-06 | -1.5E-05 | 2.4E-05 | 0.67 | | | | |
| **IL12 pg/ml** | 1.5E-04 | -9.8E-05 | 4.0E-04 | 0.23 | | | | |
| **Age at donation** | -0.012 | -0.018 | -0.007 | **< 0.001** | -0.01 | -0.02 | -0.01 | **< 0.001** |
| **BMI (kg/m²)** | 0.017 | 0.003 | 0.031 | **0.021** | 0.03 | 0.01 | 0.04 | **< 0.001** |
| **Sex** | -0.013 | -0.16 | 0.13 | 0.86 | | | | |
| **Have kids** | 0.19 | 0.016 | 0.36 | **0.032** | | | | |
| **Work with kids** | 0.21 | -0.022 | 0.45 | 0.076 | | | | |
| **Multivariable model included n = 285 with 16 missing observations deleted. Adjusted $R^2$: 0.12, *p* < 0.001.** | | | | | | | | |

*Cold frequency was natural log transformed. Est. coef = estimated coefficient, CI = confidence interval, p = p-value.*

***Different variables explain respiratory infection frequency in men and women***

Next, we assessed the differences in antiviral immune variables between men and women. IFNα production in response to RV was higher in women than in men (median = 1007.33 pg/ml vs median = 784.38 pg/ml; $p = 0.05$), and TNF and IL12 production in response to TLR8 activation was significantly lower in women (median TNF = 1967.44 pg/ml vs 2395.81 pg/ml, $p = 0.003$; median IL12 = 131.33 pg/ml vs 205.18 pg/ml, $p = 0.001$). There were no sex related differences in baseline gene expression of *TLR7, TLR8* or *CLEC4C.* Compared to younger men, older men (> 50 years) had decreased IFNα production (661.32 pg/ml vs 969.21 pg/ml, $p = 0.05$) and compared to younger women, older women (> 50 years) had decreased baseline *CLEC4C* gene expression (0.08 vs 0.12, $p = 0.03$) and increased IL12 production in response to TLR8 activation (193.71 pg/ml vs 123.43 pg/ml, $p = 0.02$) in women.

Our observations that TLR7/8 immunity varies significantly with sex and age justified the stratification of the cold frequency Model 3:1 by sex. This stratification revealed that in women, younger age and higher BMI were independent predictors of cold frequency (Model 3:2). In men, lower *TLR7* and higher *CLEC4C* gene expression were independent predictors of cold frequency (Model 3:3). In women, working with children was positively associated with cold frequency in the univariate model, but this was no longer significant in the multivariable model (Model 3:2).

*Model 3:2 Multivariable linear regression models for respiratory infection frequency in women*

| **Dependent variable: Cold frequency per year in women** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Univariate models** | | | | **Multivariable model** | | | |
| | **Est. Coef** | **CI 2.5%** | **CI 97.5%** | ***p*** | **Est. Coef** | **CI 2.5%** | **CI 97.5%** | ***p*** |
| ***CLEC4C* mRNA** | 0.40 | -0.44 | 1.2 | 0.35 | 0.23 | -0.65 | 1.11 | 0.61 |
| ***TLR7* mRNA** | -0.22 | -0.81 | 0.36 | 0.45 | -0.10 | -0.72 | 0.51 | 0.74 |
| ***TLR8* mRNA** | -0.31 | -1.0 | 0.39 | 0.39 | | | | |
| **IFNα pg/ml** | 2.5E-05 | -4.1E-05 | 9.1E-05 | 0.46 | | | | |
| **TNF pg/ml** | -3.6E-06 | -3.2E-05 | 2.5E-05 | 0.81 | | | | |
| **IL12 pg/ml** | -6.1E-05 | -5.9E-04 | 4.6E-04 | 0.82 | | | | |
| **Age at donation** | -0.016 | -0.023 | -0.009 | **< 0.001** | -0.02 | -0.03 | -0.013 | **< 0.001** |
| **BMI (kg/m²)** | 0.02 | 0.004 | 0.037 | **0.018** | 0.03 | 0.02 | 0.049 | **< 0.001** |
| **Have kids** | 0.18 | -0.042 | 0.39 | 0.11 | | | | |
| **Work with kids** | 0.29 | 0.022 | 0.56 | **0.034** | | | | |
| **Multivariable model included n = 175 with 12 missing observations deleted. Adjusted $R^2$: 0.17, *p* < 0.001.** | | | | | | | | |

*Cold frequency was natural log transformed. Est. coef = estimated coefficient, CI = confidence interval, p = p-value.*

*Model 3:3 Multivariable linear regression models for respiratory infection frequency in men*

| **Dependent variable: Cold frequency per year in men** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Univariate models** | | | | **Multivariable model** | | | |
| | **Est. Coef** | **CI 2.5%** | **CI 97.5%** | ***p*** | **Est. Coef** | **CI 2.5%** | **CI 97.5%** | ***p*** |
| ***CLEC4C* mRNA** | 0.51 | -0.15 | 1.2 | 0.13 | 0.90 | 0.19 | 1.61 | **0.01** |
| ***TLR7* mRNA** | -0.75 | -1.7 | 0.18 | 0.11 | -1.13 | -2.1 | -0.15 | **0.02** |
| ***TLR8* mRNA** | -0.096 | -0.86 | 0.67 | 0.80 | | | | |
| **IFNα pg/ml** | -3.2E-08 | -1.2E-04 | 1.2E-04 | 1.0 | | | | |
| **TNF pg/ml** | 1.3E-05 | -1.5E-05 | 4.0E-05 | 0.36 | | | | |
| **IL12 pg/ml** | 2.3E-04 | -5.2E-05 | 5.1E-04 | 0.11 | | | | |
| **Age at donation** | -0.007 | -0.016 | 0.001 | 0.086 | -0.01 | -0.016 | 6.1E-04 | 0.07 |
| **BMI (kg/m²)** | 0.007 | -0.021 | 0.034 | 0.64 | 0.02 | -0.008 | 0.050 | 0.15 |
| **Have kids** | -0.077 | -0.58 | 0.43 | 0.76 | | | | |
| **Work with kids** | 0.21 | -0.076 | 0.49 | 0.15 | | | | |
| **Multivariable model included n = 110 with 4 missing observations deleted. Adjusted $R^2$: 0.07, *p* = 0.017.** | | | | | | | | |

*Cold frequency was natural log transformed. Est. coef = estimated coefficient, CI = confidence interval, p = p-value.*

### 3.3.4. Asthma and antiviral immunity

#### *Respiratory infection frequency is increased, and baseline TLR7 gene expression is decreased in asthma*

The asthma group reported significantly more frequent respiratory infections than the healthy control group (median 3 colds per year vs 2, $p < 0.001$; Table 3:2). Because of the crucial role of TLR7 and TLR8 in detecting respiratory virus nucleic acid, we assessed TLR7/8-related antiviral immune variables for association with asthma. Out of the tested antiviral immune response variables, only *TLR7* mRNA was significantly lower in asthma

(median 0.26) than in control participants (median 0.28; $p = 0.015$; Figure 3:3). Of note, RV-induced IFNα was similar in cases and controls.

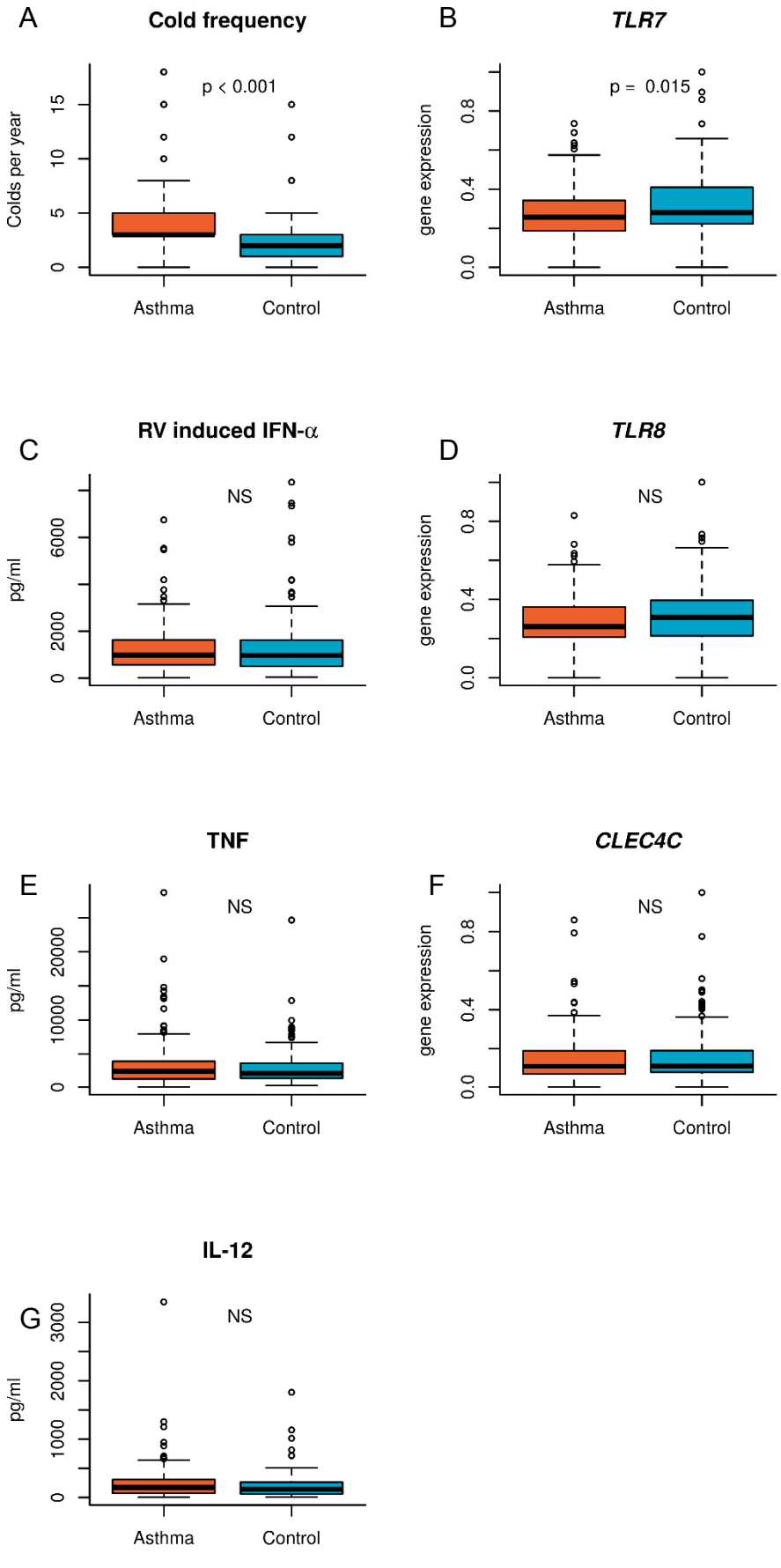
A
Cold frequency
p < 0.001
Colds per year
0
5
10
15
Asthma
Control
B
TLR7
p = 0.015
gene expression
0.0
0.4
0.8
Asthma
Control
C
RV induced IFN-α
NS
pg/ml
0
2000
6000
Asthma
Control
D
TLR8
NS
gene expression
0.0
0.4
0.8
Asthma
Control
E
TNF
NS
pg/ml
0
10000
20000
Asthma
Control
F
CLEC4C
NS
gene expression
0.0
0.4
0.8
Asthma
Control
G
IL-12
NS
pg/ml
0
1000
2000
3000
Asthma
Control

*Figure 3:3 Cold frequency and antiviral immune variables in asthma cases and controls. Median and interquartile ranges presented as boxplots for asthma and control groups for (A) number of colds per year; (B-D) RV-induced IFNα and TLR8 agonist-induced TNF and IL12 cytokine production in PBMC after 24 h stimulation; and (E-G) TLR7, TLR8 and CLEC4C gene expression in whole blood. p-value is shown for significant Mann-Whitney U-test. NS = not significant.*

Because we observed that BMI was significantly associated with increased cold frequency (Model 3:1) and the asthma group had a significantly higher BMI than controls (Table 3:2), the analysis was repeated to exclude those with BMI > 27, however the difference between the study groups remained significant (*p*-value < 0.001, Figure 3:4). BMI above 27 has been shown to indicate higher body fat composition and obesity-associated co-morbidities (Fernandez-Real et al. 2001).

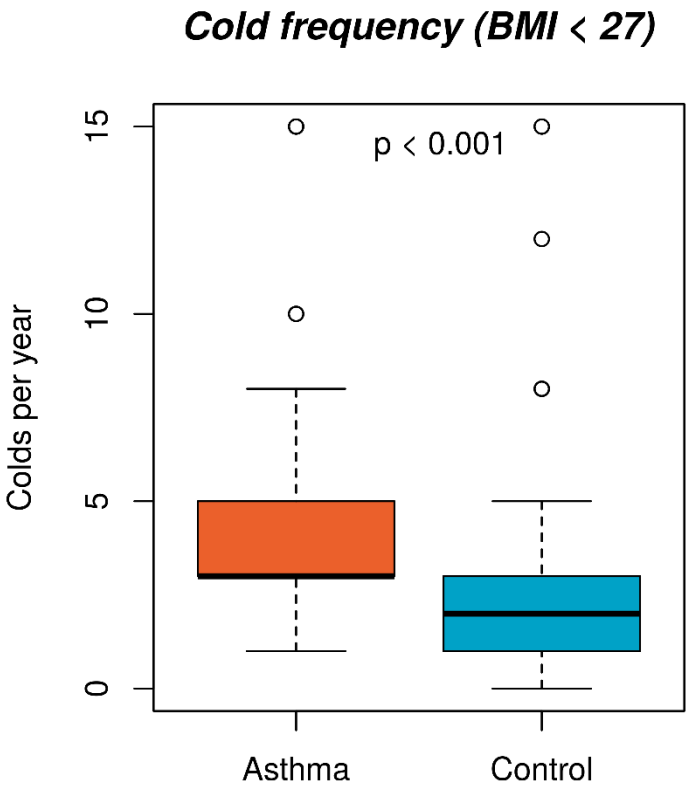


*Figure 3:4 Cold frequency between asthma cases and controls with healthy BMI. Median and interquartile ranges of respiratory infection frequency in asthma cases and controls with BMI < 27. p = p-value.*

### ***Association between asthma and antiviral variables***

Reported deficiencies in antiviral immunity in asthma in the literature warranted us to determine whether TLR7/8 related antiviral immune response variables are associated with asthma. We observed significantly lower baseline *TLR7* gene expression in asthma cases

(Figure 3:3). We further examined the variables together with a multivariable logistic regression model that was controlled for BMI, age and sex. A logistic regression model was fitted with stepwise reduction. The TLR7 association with asthma remained significant in the model when adjusted for BMI and TNF production in response to TLR8 agonist VTX-2337 (Model 3:4).

*Model 3:4 Multivariable logistic regression model for asthma.*

| **Dependent variable: Asthma** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Univariate models** | | | | **Multivariable model** | | | |
| | **OR** | **CI 2.5%** | **CI 97.5%** | ***p*** | **OR** | **CI 2.5%** | **CI 97.5%** | ***p*** |
| ***CLEC4C* mRNA** | 0.44 | 0.076 | 2.3 | 0.34 | | | | |
| ***TLR7* mRNA** | 0.12 | 0.023 | 0.62 | **0.013** | 0.12 | 0.020 | 0.69 | **0.020** |
| ***TLR8* mRNA** | 0.26 | 0.047 | 1.3 | 0.11 | | | | |
| **IFNα pg/ml** | 1.0 | 1.0 | 1.0 | 0.48 | | | | |
| **TNF pg/ml** | 1.0 | 1.0 | 1.0 | 0.31 | 1.0 | 1.0 | 1.0 | **0.050** |
| **IL12 pg/ml** | 1.0 | 1.0 | 1.0 | 0.18 | | | | |
| **Sex** | 0.81 | 0.5 | 1.3 | 0.37 | | | | |
| **Age at donation** | 1.0 | 0.99 | 1.0 | 0.34 | | | | |
| **BMI (kg/m²)** | 1.1 | 1.1 | 1.2 | **< 0.001** | 1.1 | 1.1 | 1.2 | **< 0.001** |
| **Multivariable model included n = 297 with 4 missing observations deleted. Pseudo $R^2$: 0.13, $p < 0.001$.** | | | | | | | | |

*OR = odds ratio, CI = confidence interval, p = p-value.*

### ***BMI and TNF production associate differently with asthma in men and women***

Because of the gender disparity in the prevalence of asthma and TLR7/8-related immunity, we examined the multivariable regression model for asthma in men and women separately (Model 3:5 and Model 3:6). The association between baseline *TLR7* gene expression and asthma was not significant in the separate analysis of men and women. TLR8-induced TNF production was significantly associated with asthma men but not women, whereas the BMI association with asthma was significant in women but not men, similar to the model for cold frequency described above.

*Model 3:5 Multivariable logistic regression models for asthma in women.*

| **Dependent variable: Asthma in women** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Univariate models** | | | | **Multivariable model** | | | |
| | **OR** | **CI 2.5%** | **CI 97.5%** | ***p*** | **OR** | **CI 2.5%** | **CI 97.5%** | ***p*** |
| ***CLEC4C* mRNA** | 0.75 | 0.052 | 11 | 0.83 | | | | |
| ***TLR7* mRNA** | 0.16 | 0.022 | 1.0 | 0.060 | 0.26 | 0.029 | 2.2 | 0.22 |
| ***TLR8* mRNA** | 0.15 | 0.016 | 1.3 | 0.092 | | | | |
| **IFNα pg/ml** | 1.0 | 1.0 | 1.0 | 0.29 | | | | |
| **TNF pg/ml** | 1.0 | 1.0 | 1.0 | 0.64 | 1.0 | 1.0 | 1.0 | 0.73 |
| **IL12 pg/ml** | 1.0 | 1.0 | 1.0 | 0.074 | | | | |
| **Age at donation** | 1.0 | 0.99 | 1.0 | 0.28 | | | | |
| **BMI (kg/m²)** | 1.2 | 1.1 | 1.2 | **< 0.001** | 1.2 | 1.1 | 1.2 | **< 0.001** |
| **Multivariable model included n = 184 with 3 missing observations deleted. Pseudo $R^2$: 0.17, *p* < 0.001.** | | | | | | | | |

*OR = odds ratio, CI = confidence interval, p = p-value.*

*Model 3:6 Multivariable logistic regression models for asthma in men.*

| **Dependent variable: Asthma in men** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Univariate models** | | | | **Multivariable model** | | | |
| | **OR** | **CI 2.5%** | **CI 97.5%** | ***p*** | **OR** | **CI 2.5%** | **CI 97.5%** | ***p*** |
| ***CLEC4C* mRNA** | 0.33 | 0.027 | 3.0 | 0.35 | | | | |
| ***TLR7* mRNA** | 0.067 | 0.0025 | 1.4 | 0.091 | 0.06 | 0.002 | 1.3 | 0.08 |
| ***TLR8* mRNA** | 0.56 | 0.041 | 6.7 | 0.65 | | | | |
| **IFNα pg/ml** | 1.0 | 1.0 | 1.0 | 0.78 | | | | |
| **TNF pg/ml** | 1.0 | 1.0 | 1.0 | **0.049** | 1.0 | 1.0 | 1.0 | **0.03** |
| **IL12 pg/ml** | 1.0 | 1.0 | 1.0 | 0.44 | | | | |
| **Age at donation** | 1.0 | 0.98 | 1.0 | 0.84 | | | | |
| **BMI (kg/m²)** | 1.0 | 0.96 | 1.1 | 0.28 | 1.1 | 0.98 | 1.2 | 0.14 |
| **Multivariable model included n = 113 with 1 missing observation deleted. Pseudo $R^2$: 0.12, *p* = 0.015.** | | | | | | | | |

*OR = odds ratio, CI = confidence interval, p = p-value.*

***Association between antiviral variables and different types of asthma***

Because of the wide variety of asthma symptoms in general and in our cohort (Table 3:3), we determined if asthma severity and asthma control are associated with clinical and

antiviral immune response variables. Multivariable linear regression models were used to evaluate the association between asthma severity and asthma control and antiviral variables. Baseline *TLR8* gene expression was inversely associated with ACQ6 score when adjusted for *TLR7* gene expression, BMI, and sex (Model 3:7). Baseline *TLR7* gene expression was not a significant predictor of asthma symptom control.

*Model 3:7 Multivariable linear regression model for asthma control.*

| **Dependent variable: Asthma control (ACQ6 score)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Univariate models** | | | | **Multivariable model** | | | |
| | **Est. Coef** | **CI 2.5%** | **CI 97.5%** | ***p*** | **Est. Coef** | **CI 2.5%** | **CI 97.5%** | ***p*** |
| ***CLEC4C* mRNA** | -0.071 | -1.3 | 1.1 | 0.91 | | | | |
| ***TLR7* mRNA** | -0.12 | -1.3 | 1.0 | 0.83 | 0.88 | -0.42 | 2.19 | 0.18 |
| ***TLR8* mRNA** | -1.0 | -2.1 | 0.12 | 0.079 | -1.4 | -2.7 | -0.093 | **0.036** |
| **IFNα pg/ml** | -2.4E-05 | -1.7E-04 | 1.2E-04 | 0.74 | | | | |
| **TNF pg/ml** | -2.1E-05 | -6.0E-05 | 1.7E-05 | 0.28 | | | | |
| **IL12 pg/ml** | -6.3E-05 | -5.1E-04 | 3.9E-04 | 0.78 | | | | |
| **Sex** | -0.036 | -0.35 | 0.28 | 0.82 | 0.03 | -0.28 | 0.35 | 0.84 |
| **Age at donation** | 0.016 | 0.0048 | 0.027 | **0.005** | | | | |
| **BMI ($kg/m^2$)** | 0.040 | 0.015 | 0.066 | **0.002** | 0.04 | 0.01 | 0.07 | **0.004** |
| **Multivariable model included n = 147 with 3 missing observations deleted. Adjusted $R^2$: 0.06, *p* = 0.010.** | | | | | | | | |

*Est. coef = estimated coefficient, CI = confidence interval, p = p-value.*

GINA step was treated as an ordinal variable, indicating a scale of asthma severity and analysed using ordinal logistic regression. GINA step group was significantly associated with age and BMI, but not with any antiviral immune response variables (Model 3:8).

*Model 3:8 Multivariable ordinal logistic regression model for asthma severity.*

| **Dependent variable: Asthma severity (GINA step)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Univariate models** | | | | **Multivariable model** | | | |
| | **OR** | **CI 2.5%** | **CI 97.5%** | ***p*** | **OR** | **CI 2.5%** | **CI 97.5%** | ***p*** |
| ***CLEC4C* mRNA** | 0.13 | 0.010 | 1.2 | 0.093 | | | | |
| ***TLR7* mRNA** | 0.13 | 0.015 | 1.0 | 0.057 | 0.23 | 0.024 | 2.04 | 0.19 |
| ***TLR8* mRNA** | 0.19 | 0.021 | 1.5 | 0.12 | | | | |
| **IFNα pg/ml** | 1.0 | 1.0 | 1.0 | 0.39 | | | | |
| **TNF pg/ml** | 1.0 | 1.0 | 1.0 | 0.73 | | | | |
| **IL12 pg/ml** | 1.0 | 1.0 | 1.0 | 0.36 | | | | |
| **Sex** | 1.0 | 0.53 | 1.9 | 0.98 | 1.4 | 0.70 | 2.7 | 0.36 |
| **Age at donation** | 1.0 | 1 | 1.1 | **< 0.001** | 1.0 | 1.0 | 1.06 | **0.005** |
| **BMI (kg/m$^2$)** | 1.1 | 1.1 | 1.2 | **< 0.001** | 1.1 | 1.0 | 1.1 | **0.004** |
| **Multivariable model included n = 150.** | | | | | | | | |

*OR = odds ratio, CI = confidence interval, p = p-value.*

## 3.4. Discussion

Asthma patients suffer from worse respiratory infections; however, it is unclear if they are infected more often and what kind of clinical and immunological factors play a role in infection susceptibility. This study examined clinical and immunological parameters associated with self-reported respiratory infection frequency, the presence of asthma, asthma severity and control, with special focus on the role of TLR7/8 gene expression and function. We wanted to further determine to what extent these associations differ between women and men.

Here we show that in men, self-reported respiratory infection frequency is associated with reduced baseline *TLR7* gene expression and increased relative pDC quantity, and in women, self-reported respiratory infection frequency is associated with increased BMI and younger age. Participants with asthma reported more frequent respiratory infections and had reduced *TLR7* gene expression in comparison to controls. Asthma severity, as reflected in treatment intensity, was not associated significantly with immunological variables, but poor asthma symptom control was associated with reduced TLR8 gene expression and increased BMI.

We assessed several TLR7/8-related antiviral immune variables for an association with respiratory infection frequency and detected a trend towards an inverse correlation with baseline *TLR7* gene expression, *i.e.* those with the lowest TLR7 expression tended to have a higher frequency of self-reported respiratory infections. The weak correlation implies that TLR7's role in virus recognition may be necessary for preventing respiratory infections. The multivariable linear regression analysis showed that the gene expression of the pDC marker *CLEC4C*, BMI and age independently predicted respiratory infection frequency. However, further stratification of the model by gender revealed that different variables are associated in men and women.

In men, increased *CLEC4C* in conjunction with decreased *TLR7* gene expression was associated with high respiratory infection frequency. This result is somewhat unexpected, as pDC are thought to be the primary cell type that expresses TLR7. Hence the association between *CLEC4C* expression and infection frequency was expected to be in the same direction as the association between TLR7 gene expression and infection frequency. Others have reported that TLR7 induced IFNα production is less efficient in men than in women but

is independent of pDC numbers (Berghofer et al. 2006). Our results suggest a similar effect in men, where the inverse correlation between TLR7 and respiratory infection frequency appears to be independent of pDC numbers. Although pDC play an essential role in antiviral immunity, high pDC numbers have been reported in adults with asthma (Spears et al. 2011) and are further elevated in response to allergen challenge (Dua et al. 2010). In contrast, lower numbers of blood pDC in infancy is a risk factor for more frequent respiratory tract infections, wheezing, and a subsequent diagnosis of asthma (Upham et al. 2009). The association with allergies and asthma may indicate that the functionality of pDC is different between asthma and controls, and the relative pDC numbers may not fully reflect pDC antiviral immune function. We did not see a difference in *CLEC4C* gene expression between asthma and healthy groups (Figure 3:3), yet TLR7 gene expression was lower, supporting an asthma-related reduction in pDC functionality.

Another explanation for the opposite direction of effect for *CLEC4C* and *TLR7* gene expression is that as the baseline gene expression was measured in whole blood, and many other cell types than pDC could influence *TLR7* gene expression. TLR7 is expressed by eosinophils in asthma and appears to alter their function (Mansson & Cardell 2009). Furthermore, eosinophils have recently been recognised to capture viruses, a function that is impaired in asthma patients (Sabogal Piñeros et al.). It is, therefore, possible that a reduction in *TLR7* gene expression across all the TLR7 expressing immune cells could influence cold susceptibility.

In contrast, only young age and high BMI were significant independent predictors of higher cold frequency in women, with none of the immune function parameters being associated with cold frequency. Younger women are most likely to be in contact with small children, a known infection risk (Galanti et al. 2019); however, self-reported contact with children was not associated with cold frequency in our final fitted regression model. Protective immune memory associated with increased age could explain the association with age observed in women. Another explanation for the difference between sexes could be hormonal changes associated with being overweight and age, particularly in women. Substituting age measured as a continuous variable with a binomial variable using 50 years of age as a cut-off in the models maintained near identical effect and significance (not shown), suggesting that hormonal changes associated with menopause may explain the association with age. The female hormone oestrogen declines with age and is higher in obese people, which here

would suggest that lower oestrogen levels are beneficial. Oestrogen has been shown to modify pDC responses through TLR7 (Seillet et al. 2012) and both TLR7/8 expression (Young et al. 2014). The relationship between TLR7 function and oestrogen suggests that female sex hormones, known to be influenced by BMI and age, might obscure any association between TLR7 and respiratory infection frequency. We did not collect data on hormonal contraceptive use, but as it is commonly used in young women, it may explain the age association with cold frequency (Scott, H. A. et al. 2016). In hindsight, it would have been interesting to have collected information from female participants on the timing of blood collection in relation to their individual menstrual cycles. Although obese women have higher oestrogen levels, there are also changes in progesterone and androgen levels that contribute to abnormal menstrual cycles (Escobar-Morreale et al. 2017; Lash & Armstrong 2009) and which might modify antiviral immunity. Obesity is linked with impaired immune function, including altered T- and B-cell populations, altered immune responses and systemic inflammation (Karlsson & Beck 2010; Sheridan et al. 2011). Obese people are more susceptible to influenza infections (Karki et al. 2018; Louie et al. 2011), and overweight children are susceptible to respiratory infections (Jedrychowski et al. 1998), and our results indicate a similar association in adult women.

Corne et al. (2002) followed cohabiting couples consisting of one partner with asthma and one without asthma for three months and those with asthma had more prolonged lower respiratory symptoms, though they found no difference in the *frequency* of RV infections. In contrast, our study participants with asthma reported more frequent respiratory infections than non-asthmatic participants. The frequency of respiratory infections was self-reported as an estimated number of illnesses suffered per year. A note of caution is due here since the study may have attracted participants who suffer from frequent respiratory infections; however, the median frequency of 2 colds per year in the healthy controls is in line with other reports (Leder et al. 2003). Additionally, recalling the number of past infections could be biased by factors like the severity of the experienced illnesses, gender, psychological factors, or motivation by the topic of the study. However, the large sample size of 301 used in this study is expected to mitigate some of these factors. Importantly, this study identified several biological variables that were independently associated with respiratory infection frequency, suggesting that self-reported colds has some validity as a relevant outcome. We suspected that obese participants might have influenced the result, however when we

repeated the analysis with stratification for obesity, the outcome remained the same, confirming that the difference is not due to BMI imbalance between the groups.

Subsequently, we evaluated antiviral immunity in asthma. Many studies report deficient type I IFN production in response to viral infection in asthma (as discussed in Chapter 1, section 1.4.2). In contrast with the previous reports of others and our own group (Edwards et al. 2017; Pritchard et al. 2014), we did not observe lower IFNα production in response to RV in the asthma group in comparison to the control group. The sample size in this study was much larger than many previous studies, and we followed a similar protocol to others. However, it is possible that the long duration of two years for recruiting and performing the experiments could have introduced technical variables in the study. To minimise such variables, asthma and control participants were recruited simultaneously. Moreover, it is possible that IFN deficiency may be observed best at a different time point rather than the 24 h post-stimulation time point used by this study and by others (Durrani et al. 2012; Gehlhar et al. 2006). It was not practical to assess IFNα production at multiple time points in a study of this size, nor was it feasible to study lung tissue.

Immune responses are known to vary with season (ter Horst et al. 2016), which may affect IFNα responses too. Due to the higher susceptibility and worse cold infections, many of our asthma participants were sick during the winter months. Despite waiting at least two weeks after cold symptoms before sampling, the cold might have affected their immune responses. Another consideration is the possible effect of asthma symptoms. While most studies agree that IFN production is either lower or similar in asthma cases and controls, there are reports that IFNλ production is higher during asthma exacerbations in children (Miller et al. 2012), and IFNβ production in response to RV increases in IL4 and IL13 co-culture (Herbert et al. 2017). Transcriptome studies of asthma exacerbation types reveal distinct asthma subtypes where IFN production during an exacerbation is either high or low (Gomez et al. 2018; Khoo et al. 2019). An elaborate study by Custovic et al. (2018) identified several possible clusters of different cytokine responses to RV in year old children and searched for associations with childhood asthma. Similar to the transcriptome studies (Gomez et al. 2018; Khoo et al. 2019), they identified two clusters with the lowest and highest IFN response that were associated with asthma. These controversies between studies emphasise the fact that type I IFN deficiency probably exists in a subgroup of asthma patients, however high IFN production is also linked with asthma. In conjunction, the literature and our findings indicate

that the phenotypes associated with type I IFN response variation are not explicit and are most likely influenced by several variables. Our finding that IFNα was similar in those with asthma and healthy participants was not anticipated. However, we did observe great variability in IFNα response across the participants. We questioned what factors beyond asthma-related attributes associate with the intensity of IFNα response to RV stimulation. The differences between high and low IFNα responders will be examined further in Chapter 4.

Rather than type I IFN deficiency, we saw that the baseline TLR7 gene expression was lower in the asthma group, which remained significant in the fitted multivariable linear regression model for asthma. The association was not significant in the separate analysis of men and women, though this may be because the sample size was insufficient. TNF production and BMI were also associated with asthma; however, the effect size of TNF was minimal. Reduced TLR7 function in asthma is well documented: Pritchard et al. (2014) showed lower *TLR7* gene expression in asthmatics in response to RV16 stimulation, along with other genes in the TLR7 signalling pathway; in an allergic mouse model, reduced TLR7 activity was the result of Th2-immunity; and in humans lower TLR7 expression in asthma lungs correlated with sputum eosinophilia (Hatchwell et al. 2015). The consequence of impaired TLR7 function in asthma may be frequent or severe respiratory infections. These results suggest that altered antiviral immune responses in asthma may occur *upstream* of IFNα production. Indeed, genomic analysis of antiviral immune response pathways showed that apart from 5–6 core signalling factors, the pathways leading to the detection of pathogens and immune response are highly flexible (Troy & Bosco 2016), indicating that deficiencies may occur at several levels. Genetic variation in the TLR7 gene region has been reported to be associated with asthma, and genetic variation could explain the lower TLR7 gene expression asthma group that we observed and lack of association with IFN production. The consequences of TLR7/8 gene region are studied in Chapter 5.

Asthma is recognised as a heterogeneous disease consisting of endotypes with varied clinical presentation (Deliu et al. 2016). It is therefore likely that deficiencies in antiviral immunity are not global across all asthma patients, but specific to certain endotypes. We recruited participants with a spectrum of asthma severities and symptom control scores and used multivariable linear regression analysis to evaluate the association between several TLR7/8-related antiviral immune variables and asthma severity and control. Asthma control

was best explained by a multivariable linear regression model that included *TLR7* and *TLR8* gene expression, sex and BMI. We expected to see lower TLR7 gene expression associated with poor asthma symptom control. In contrast, we saw reduced TLR8 gene expression. The central function of the cytokine repertoire induced by TLR8 is the promotion of Th1-polarised immune responses (Lu et al. 2012). Because type 1 immunity antagonises type 2 immunity, reduced TLR8 gene expression seen in asthma patients with worse asthma symptom control is not surprising. Although a crossregulatory relationship exists between TLR7 and TLR8 receptors, possibly via competing for a common chaperone protein Unc93B1 (Desnues et al. 2014; Itoh et al. 2011), very few cells express both receptors and one of the advantages of using multivariable linear regression analysis is to explore confounding effects from other variables. The regression model included *TLR7* gene expression, indicating that *TLR8* gene expression is associated with asthma symptom control independently of the effect of *TLR7* gene expression.

Pinpointing the asthma subgroup that is at most risk for deficiencies in antiviral immunity is vital for improving asthma treatment. Our study was limited to self-reported features for both asthma symptoms and respiratory infections. Nevertheless, the increased number of self-reported respiratory infections in the asthma population highlights the burden of respiratory infections that people with asthma experience. Our results show that while TLR7 gene expression at baseline is reduced overall in the asthma group, this reduction is not associated with severe asthma or poor asthma control. In contrast, poorly controlled asthma patients had reduced TLR8 baseline gene expression. Our results raise the questions whether *TLR7/8* gene expression is reduced by excessive type 2 immunity and what consequences reduced TLR7/8 function has on antiviral immune parameters such as cytokine response and infection severity and duration. A thorough analysis of different asthma endotypes defined by sputum markers could elucidate what asthma phenotypes drive the TLR7/8 deficiency or are associated with such deficiency. Our findings and the prospect of treating asthma with TLR7/8 agonist medications under development emphasise the importance of continuing research on TLR7/8 in asthma.

Finally, the observation that the asthma group had a higher BMI than controls requires mention. BMI remained a significant predictor in all the linear regression models. Obesity is a risk factor for asthma development and is associated with more severe disease, while weight loss improves asthma symptoms (Scott, Hayley A. et al. 2015; Wood 2017). In

addition to asthma risk, obesity has been associated with an increased risk of respiratory infection and a decline in antiviral immunity (Karlsson & Beck 2010), raising the question as to whether obese asthmatics are at more risk of respiratory infections than normal-weight asthma patients. Obesity and asthma are associated with systemic inflammation and, in combination, contribute to changes in cytokine environment with a potentially detrimental effect on antiviral immunity. A shortcoming of our study was unmatched study groups regarding BMI, and we recommend considering BMI in both asthma and antiviral immunity studies at the study design phase. Investigating these relationships is essential, as achieving and maintaining a healthy weight may lead to vast improvements in both asthma symptoms and respiratory infection-related problems.

In conclusion, the results presented in this chapter show a role for TLR7/8-related immunity in respiratory infection frequency and asthma. The predictors of frequent respiratory infections were different in men and women. Men are known to respond weakly to RV infections (Carroll et al. 2010), and our results imply that men are more sensitive to variation in antiviral immune response variables, whereas women are susceptible to the influence of BMI and age. BMI influenced all of the outcomes that we analysed, suggesting a role in both asthma and antiviral immunity.

# Chapter 4: Transcriptional variation predicting a robust interferon response to rhinovirus challenge

## 4.1. Introduction

Type I interferons (IFN) are vital cytokines that induce an antiviral response upon viral encounter. This chapter investigates the transcriptome, and how this is related to variations in capacity for type I IFN production in response to rhinovirus (RV).

Type I IFNs have a dual role in activating the antiviral immune response. Initially, they induce an antiviral state in infected cells and activate the innate immune system, recruiting and stimulating antigen presenting cells (APCs) and natural killer (NK) cells. As a secondary antiviral response, type I IFN also directly activates adaptive immune cells such as and T- and B- lymphocytes to differentiate into pathogen-specific effector and memory cells (Crouse, Kalinke & Oxenius 2015; Levy, Marié & Durbin 2011). The subgroup of type I IFNs consists of several subtypes, but predominantly thirteen interferon alpha (IFNα) and one interferon beta (IFNβ). While almost all cells secrete IFNβ and many immune cells secrete IFNα, plasmacytoid dendritic cells (pDC) are the main producers of type I IFN (Xi et al. 2015). The shared IFN α/β receptor (IFNAR) is expressed on all nucleated cells to enable their response to type I IFN. Signalling through IFNAR triggers the expression of IFN stimulated genes (ISG) that induce an antiviral state in the cell and to produce cytokines and chemokines to attract immune cells.

Type I IFN is produced constitutively in low, almost undetectable amounts (Gough et al. 2012), and is augmented only in response to an immune stimulus, such as exposure to viruses. Pathogen recognition receptors (PRRs), such as toll-like receptor (TLR)7 and TLR8, detect viral particles that activate the antiviral immune response within which type I IFN production plays a crucial role. Genetic diseases that alter the type I IFN response, whether deficiency or abundance, result in severe pathologies, or at worst, an incompatibility with life (Taft & Bogunovic 2018). Sufficient type I IFN production is integral to an efficient antiviral immune response and correlates with viral replication in epithelial cells in vitro, and with viral load and disease severity in vivo (Edwards, MR et al. 2013; Hayden et al. 1998). Within the healthy range of type I IFN production, variation is known to arise from factors such as gender, age and hormones (Berghofer et al. 2006; Metcalf et al. 2015; Seillet et al. 2012).

In Chapter 3, we observed a broad interindividual variation in IFNα response to RV stimulation. Previous literature highlights an optimal range of type I IFN production for eliciting an efficient antiviral immune response and protecting from inflammation (Taft &

Bogunovic 2018). Immune variation within healthy individuals influences the risk of immunopathologies, infections and poor vaccine response. In the era of high-throughput research, several researchers have taken advantage of various ‘omics technologies to study immune variation. This relatively new field of study provides the insight that genetics together with environment and host factors such as age, gender, immune cell frequency, microbiome and cohabitation, influence immune variation (Bakker et al. 2018; Brodin et al. 2015; Carr et al. 2016; Kaczorowski et al. 2017; Lee, MN et al. 2014; Piasecka et al. 2018; Schirmer et al. 2016; ter Horst et al. 2016). Despite the extent of these studies, their ability to explain variations in type I IFN response to respiratory viruses is incomplete. Lee, MN et al. (2014) and Piasecka et al. (2018) have comprehensively studied the antiviral response to influenza virus A (IAV), however, the distinct host antiviral immune response to IAV may not translate to other respiratory viruses such as RV (Troy & Bosco 2016).

Therefore, the experiments described in this chapter examine the extent to which transcriptional variations predict the antiviral type I IFN responses, comparing transcriptomes between individuals who exhibit very high and very low antiviral type I IFN responses. Knowledge about this topic is currently limited. Though Chapter 3 showed no clear difference in type I IFN production between asthmatic and healthy groups (Chapter 3), baseline gene expression levels of TLR7 were significantly lower in the asthma group than controls, so we also examined the extent to which transcriptomes differed between healthy people and those with asthma.

Whole transcriptome analysis enables the study of total gene expression in cells and thus provides a tool for understanding the biological differences between conditions. To address our research questions, we selected participants with contrasting IFNα production in response to RV16 stimulation and compared their whole transcriptomes at baseline in unstimulated peripheral blood mononuclear cells (PBMC) and during the antiviral response in RV16-stimulated PBMC. Contrasting IFNα producers will help understand underlying host and immunological factors contributing to differences in IFNα response.

## 4.2. Materials and methods

### 4.2.1. Participant selection

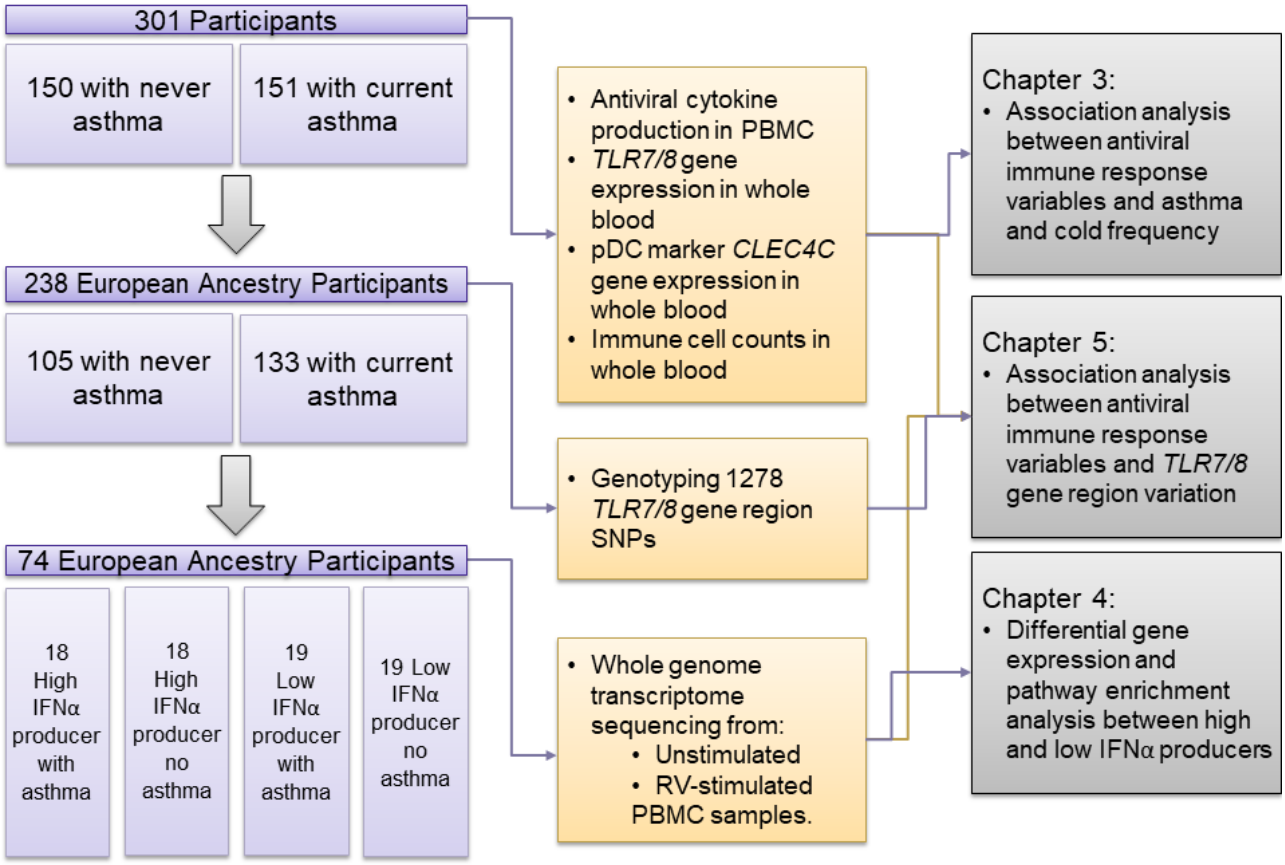


*Figure 4:1 Study design.*
*Samples selected for the current study were selected from an asthma case study.*

Cytokine production in PBMC in response to stimulation with medium (control), 5μM TLR7 specific agonist Imiquimod (Invivogen, USA) and RV16 were measured as a part of the experiments in Chapter 3, and the methods are described in section 3.2.4. In the recruited cohort of 301 people, we observed that certain individuals responded to both imiquimod and RV16 stimulus with highly efficient IFNα production, whereas others responded with a low amount of IFNα (Figure 4:2). Participants were part of the cohort described in Chapter 3 (section 3.2.1). Clinical features were recorded, and blood cell counts were measured as described in Chapter 3: section 3.2. Two contrasting IFNα producing groups were selected from 238 samples from participants with European ancestry (Figure 4:1). Seventy-four samples in the highest 15% or lowest 15% of IFNα response to RV16 stimulation in PBMC

were selected for the current study. Thirty-six samples in the high IFNα producer group comprised 18 asthma and 18 healthy samples, and 38 samples in the low IFNα producer group comprised 19 asthma and 19 healthy samples. The high and low IFNα producer groups will be referred to as IFN-high and IFN-low groups from here on.

### 4.2.2. Quantification of whole blood gene expression with RT-PCR

The methods for quantifying gene expression of *TLR7, TLR8* and *CLEC4C* in the unstimulated condition in whole blood with RT-PCR are described in Chapter 2: section 2.2.5 and Chapter 3: section 3.2.4.

### 4.2.3. Sequencing of RNA extracted from PBMC

RNA in PBMC samples was preserved in RNAprotect™ (Qiagen, Hilden, Germany) as per manufacturers' instructions, and stored at -80°C. Samples were extracted for total RNA and sequenced at Macrogen Inc (Seoul, Rep. of Korea) with Truseq mRNA kit using the NovaSeq6000 platform, with a minimum of 20 million, 100bp paired-end reads per sample. Two PBMC RNA samples were sequenced per individual: one unstimulated (baseline) and one RV16-stimulated (infection) for 24 h.

### 4.2.4. Alignment of sequence data

The Genome Informatics Group at QIMR Berghofer, Queensland, Australia, aligned the sequence reads. Sequence reads were trimmed for adapter sequences using Cutadapt [version 1.11; (Martin 2011)] and aligned using STAR [version 2.5.2a; (Dobin et al. 2013)] to the GRCh37 assembly with the gene, transcript and exon features of Ensembl [release 89; (Zerbino et al. 2017)] gene model. Quality control metrics were computed using RNA-SeQC [version 1.1.8; (DeLuca et al. 2012)] and gene expression levels were quantified using RSEM [version 1.2.30; (Li & Dewey 2011)].

### 4.2.5. Differential gene expression analysis

EdgeR package (Robinson, MD, McCarthy & Smyth 2010), was utilised for the discovery of differentially expressed genes in IFN-high group compared with IFN-low group. Library size was corrected using counts per million (CPM), which involves dividing each sample gene count by the total number of mapped reads. Genes with CPM > 2 in more than six samples were retained for analysis. Trimmed mean of M-values was used to normalise differences

in RNA composition between samples with the function *calcNormFactors()* from the edgeR package. Function *glmQLFit()* fits a quasi-likelihood negative binomial generalised log-linear model to count data. Significant genes were filtered with multiple testing corrected false discovery rate (FDR) < 0.05 and log fold-change > 1. RV16-stimulated and unstimulated samples were tested separately.

***Biological pathway analysis***

Identification of significantly enriched biological pathways was performed using gene set enrichment analysis (GSEA) (Subramanian et al. 2005) focussing on genes differentially expressed between IFN-high and IFN-low groups at baseline and with RV16-stimulation. Fold change in gene expression was used for ranked list of input to GSEA, and gene ontology (GO) pathways with FDR < 0.25 were considered as biological pathways significantly related to the differentially expressed genes.

### 4.2.6. Statistical analysis

All statistical analyses were performed with R version 3.4.4 (R Core Team 2018). Variables were tested for normality and consequently, treated as nonparametric variables. *CreateTableOne()* function in the R package tableone was used to create the demographics table of the study group and perform statistical tests (Yoshida 2019). *p*-value < 0.05 was considered significant. The difference in sample distribution was tested with a nonparametric Mann-Whitney *U* test, which was considered significant with *p*-value < 0.05 / 6 tests = 0.008 after multiple testing correction in section 4.3.2, and *p*-value < 0.05 / 4 tests = 0.013 after multiple testing correction in section 4.3.4. Paired nonparametric Spearman's rank correlation test was used to test correlation between observations. Correlation with a *p*-value < 0.05 was considered statistically significant. In section 4.3.4 the expression of 11 IFNα genes that were quantified (*IFNA4, IFNA7, IFNA10, IFNA16, IFNA17, IFNA13, IFNA2, IFNA8, IFNA1, IFNA14* and *IFNA5),* were summed for statistical analysis.

***Graphs***

Boxplots and before-after plots were created with GraphPad Prism software (version 7, La Jolla, USA; Figure 4:2 and Figure 4:5). Principal component analysis (PCA) was performed with *prcomp()* function and plotted using *autoplot()* function from ggfortify package [Figure

4:3 PCA analysis of unstimulated samples. and Figure 4:4; (Tang, Horikoshi & Li 2016)]. GSEA pathway plots were created with *ggplot()* function in ggplot2 package [Figure 4:7 and

Figure 4:10 ; (Wickham 2016)], GO-pathway networks were generated in Cytoscape interface [San Diego, USA, (Shannon et al. 2003)], and heatmaps were created with Pheatmap package [Figure 4:6 and Figure 4:9; (Kolde 2019)] with base function *hclust()* clustering and dendrogram sorting with dendsort package (Sakai 2015).

### 4.2.7. Access to public databases

We searched the manually annotated records in the protein knowledgebase UniProt (The UniProt Consortium 2018) and PubMed (NIH, USA) for any additional literature supporting the understanding of the functions of the gene products.

## 4.3. Results

### 4.3.1. Variation in type I interferon production

Of the 301 participants whose cells were stimulated with TLR7 agonist and RV16 for 24 h in Chapter 3, we found that RV16 stimulation elicited the broadest interindividual variation in IFNα production in PBMC (median 969.2; IQR 529, 1633) (Figure 4:2:A). IFNα production in response to TLR7 agonist and RV16 were highly correlated ($r = 0.66$, $p < 0.001$), and to maximise the number of samples analysed within economical constraints, we chose to compare only unstimulated and RV16-stimulated PBMC samples. We subsequently selected 74 participants with the highest IFNα and lowest IFNα production in each group to compare differences in gene transcription associated with extreme phenotypes of IFNα production as visualised in Figure 4:2:B.

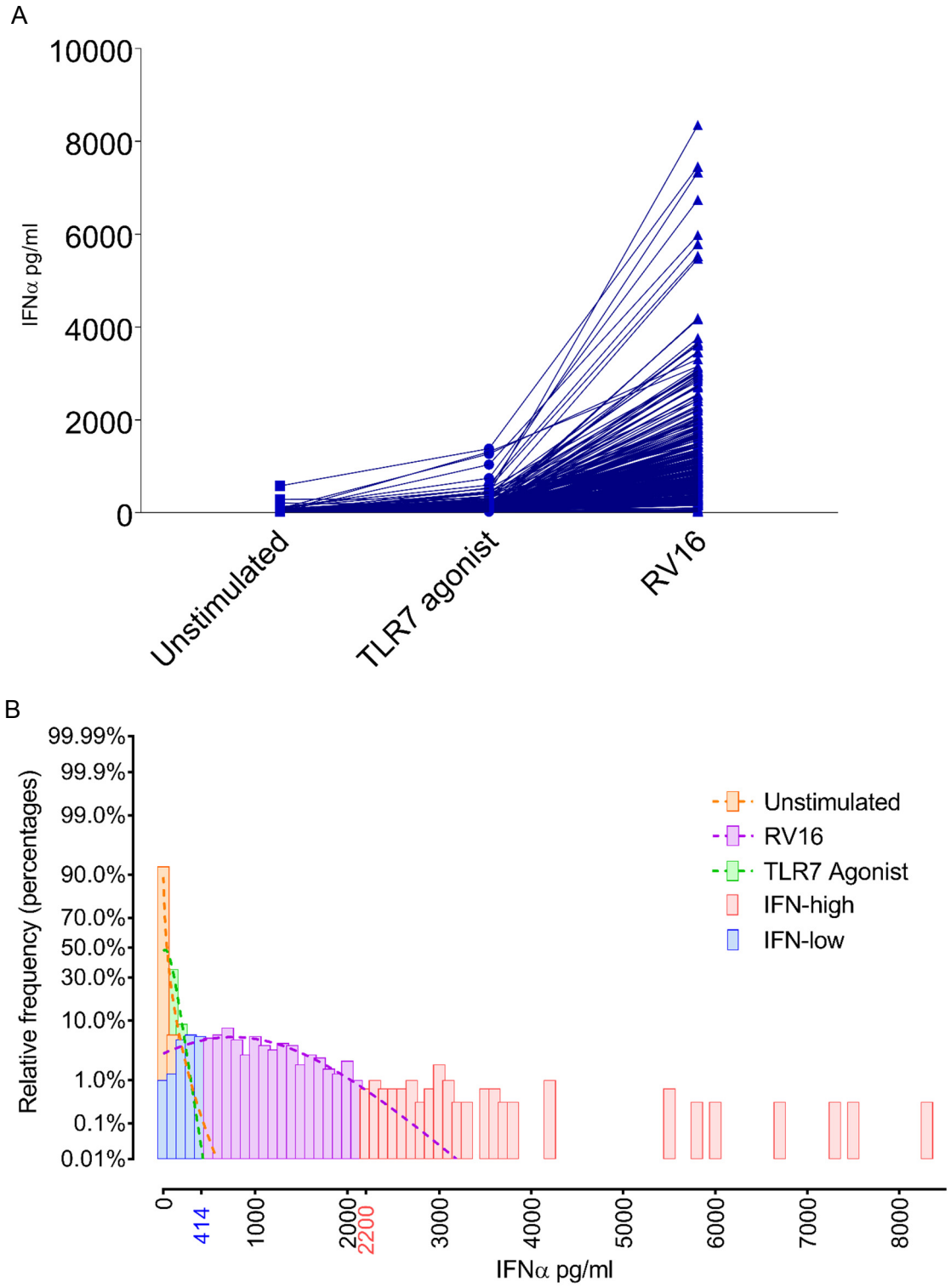


*Figure 4:2 Variation in IFNα production by the stimulus.*
*A) Paired IFNα produced in PBMC after 24 h of no stimulation (control), TLR7 agonist (imiquimod) or rhinovirus-16 (RV) stimulation. B) Relative frequency of IFNα production in unstimulated PBMC or in response to RV16 or TLR7 agonist. Blue RV16-stimulated samples with IFNα production below 414 pg/ml were chosen as IFN-low samples, and red samples with IFNα production above 2200 pg/ml were selected as the IFN-high samples. Dotted lines show the frequency distribution of each stimulation fitted to a Gaussian distribution.*

### 4.3.2. The demographics of the study groups

Table 4:1 compares the differences in clinical and molecular features between high and low IFNα producers within the asthma and healthy control groups. The primary selection criteria for this study was high or low IFNα production. Participants were selected separately from asthma and control groups to maintain equal sample size, but omitted adjustment for sex, age and BMI to preserve the contrast between the groups. The results show that the IFN-low group (n = 38) had significantly higher BMI (median 23 vs 26; $p$ = 0.001) than the IFN-high group (n = 36), and the IFN-low group tended to be slightly older, though this was not statistically significant ($p$ = 0.053). However, between asthmatic participants, low IFNα producers were significantly older than the high producers ($p$ = 0.004). There were no significant differences in asthma control, severity, or frequency of respiratory infections between IFN-high and IFN-low groups. Sixty-five per cent were female; however, stratification by group reveals that the healthy IFN-low group has fewer females at 50%, however not significantly fewer in comparison to other groups (Table 4:1).

*TLR7* and *TLR8* gene expression as measured in unstimulated conditions in whole blood were similar between the groups; however, the pDC cell marker *CLEC4C* gene expression was significantly lower in the IFN-low group ($p$-value < 0.001) suggesting relatively lower pDC cell count (as discussed in Chapter 2). TLR8-activated TNF production was also lower in the IFN-low group ($p$-value = 0.006), whereas TLR8-activated IL12 production was similar in both groups. Peripheral blood granulocytes and smoking status were similar in both groups; only eosinophils were higher in the asthma groups ($p$-value = 0.003).

*Table 4:1 Characteristic of the study groups classified by asthma status.*

| | IFN-high | | IFN-low | | |
|---|---|---|---|---|---|
| | **Asthma** | **No** | **Asthma** | **No** | ***p*** |
| **n** | 18 | 18 | 19 | 18 | |
| **Female (%)** | 13 (72.2) | 13 (72.2) | 13 (68.4) | 9 (50.0) | 0.438 |
| **Age at donation** | 27.00 [24.00, 34.50] | 32.50 [26.00, 43.75] | 47.00 [35.50, 53.00] a | 36.50 [23.25, 49.25] | **0.045** |
| **BMI kg/m²** | 23.00 [21.25, 24.00] | 23.50 [21.00, 26.00] | 27.00 [25.00, 30.50] a | 24.50 [24.00, 27.00] | 0.008 |
| | IFN-high: 23.00 [21.00, 25.25] | | IFN-low: 26.00 [24.00, 30.00] d | | |
| **GINA step (%)** | 7 (38.9) | | 6 (31.6) | | 0.391 |
| | 1 (5.6) | | 0 (0.0) | | |
| | 1 (5.6) | | 3 (15.8) | | |
| | 9 (50.0) | | 8 (42.1) | | |
| | 0 (0.0) | | 2 (10.5) | | |
| **ACQ score** | 0.50 [0.17, 0.96] | | 0.83 [0.25, 1.92] | | 0.271 |
| **Cold frequency** | 3.00 [3.00, 5.00] | 3.00 [1.00, 3.00] | 3.00 [3.00, 5.00] | 1.50 [1.00, 3.00] c | **0.004** |
| | All asthma: 3.00 [3.00, 5.00] | | All Healthy: 2.00 [1.00, 3.00] e | | |
| **Gene expression in whole blood** | | | | | |
| ***TLR7*** | 0.29 [0.24, 0.44] | 0.26 [0.21, 0.33] | 0.25 [0.20, 0.35] | 0.28 [0.21, 0.36] | 0.596 |
| ***TLR8*** | 0.28 [0.23, 0.32] | 0.28 [0.22, 0.39] | 0.25 [0.20, 0.35] | 0.34 [0.24, 0.46] | 0.198 |
| ***CLEC4C*** | 0.19 [0.10, 0.26] | 0.15 [0.10, 0.22] | 0.10 [0.06, 0.12] | 0.08 [0.06, 0.11] | **0.006** |
| | IFN-high: 0.15 [0.10, 0.26] | | IFN-low: 0.08 [0.06, 0.11] d | | |
| **Cytokine production in PBMC** | | | | | |
| **IFNα pg/ml (RV-stim.)** | 3011.11 [2538.10, 3652.04] | 3265.26 [2887.05, 4044.42] | 297.66 [279.42, 346.75] a | 223.58 [190.87, 301.19] b | **<0.001** |
| | IFN-high: 3068.18 [2736.95, 3866.79] | | IFN-low: 279.42 [196.65, 324.94] d | | |
| **TNF pg/ml (TLR8-stim.)** | 3799.00 [2347.82, 5515.34] | 2354.01 [927.12, 4174.55] | 1654.43 [667.49, 3439.06] | 1829.32 [909.25, 2135.81] | **0.016** |
| | IFN-high: 3180.04 [1827.26, 4841.36] | | IFN-low: 1798.82 [754.18, 3082.33] d | | |
| **IL12 pg/ml (TLR8-stim.)** | 196.74 [113.06, 291.70] | 99.17 [26.67, 144.82] | 202.04 [61.75, 288.57] | 103.07 [72.66, 184.12] | 0.102 |
| **White blood cells x $10^9$/L** | 6.35 [5.62, 7.65] | 6.60 [5.05, 7.40] | 6.20 [5.25, 7.65] | 5.80 [4.90, 8.22] | 0.866 |
| **Neutrophils x $10^9$/L** | 3.45 [2.87, 4.41] | 3.78 [2.66, 4.99] | 3.48 [2.92, 4.17] | 3.16 [2.82, 5.37] | 0.924 |

| | IFN-high | | IFN-low | | |
|---|---|---|---|---|---|
| | **Asthma** | **No** | **Asthma** | **No** | ***p*** |
| **Eosinophils x $10^9$/L** | 0.23 [0.12, 0.38] | 0.15 [0.08, 0.24] | 0.26 [0.16, 0.33] | 0.11 [0.08, 0.17] c | **0.003** |
| | All asthma: 0.25 [0.14, 0.36] | | All healthy: 0.12 [0.08, 0.19] e | | |
| **Pack years smoked** | 0.00 [0.00, 0.00] | 0.00 [0.00, 0.00] | 0.00 [0.00, 0.00] | 0.00 [0.00, 0.00] | 0.899 |

*n = study group size; TLR8-ag. stim. = TLR8 agonist VTX-2337 stimulated. Variables are expressed as median [interquartile range] or n observations (%). GINA step measures asthma severity and ACQ score asthma control score. The bold p-value indicates statistically significant Kruskal-Wallis ranks sum test between the four groups. Significant post-hoc Mann-Whitney U-test indicated as: [a]p < 0.008 vs IFN-high asthma group; [b]p < 0.008 vs IFN-high healthy group; [c]p < 0.008 vs IFN-low asthma group; [d]p < 0.008 vs IFN-high group; [e]p < 0.008 vs asthma groups.*

### 4.3.3. Principal component analysis of transcriptomes

We compared the transcriptomes between the asthma and healthy IFN-high and IFN-low groups. Principal component analysis (PCA) of baseline and RV16-stimulated sample transcriptomes indicated a modest grouping of samples by IFNα producer groups across PC1 (unstimulated Figure 4:3, RV16-stimulated Figure 4:4). Because PCA revealed no apparent distinction between asthmatic and non-asthmatic samples, this warranted a pooled analysis of the IFN-high and IFN-low groups, regardless of asthma status, to address the primary objective of the study.

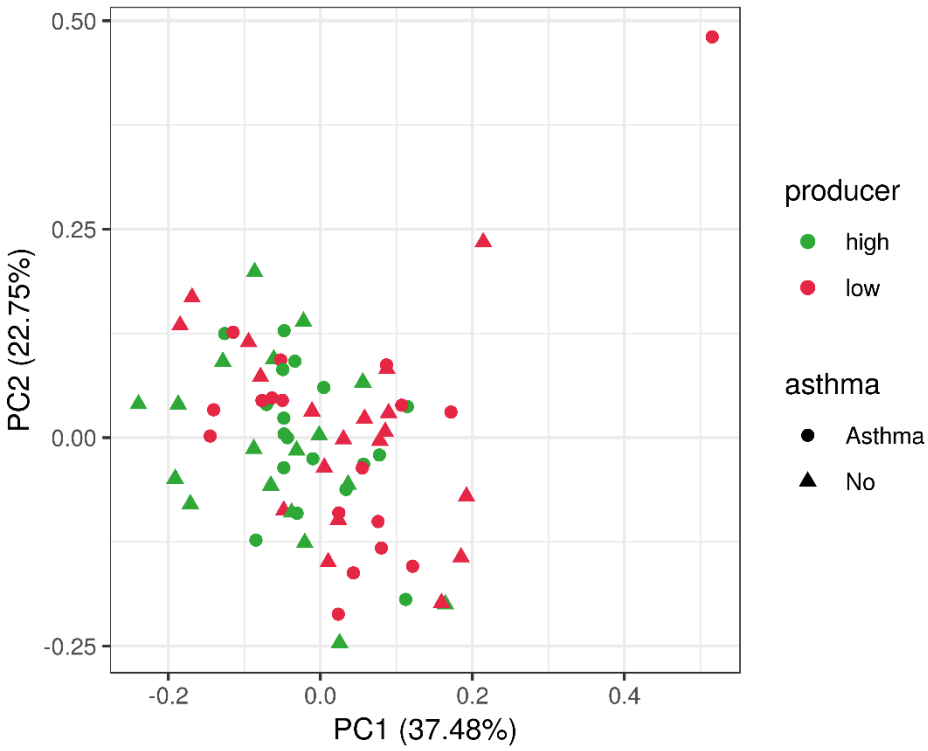


*Figure 4:3 PCA analysis of unstimulated samples.*
*Green indicates high and red indicates low IFNα producer samples. Round shapes indicate asthma and triangle shapes healthy samples.*

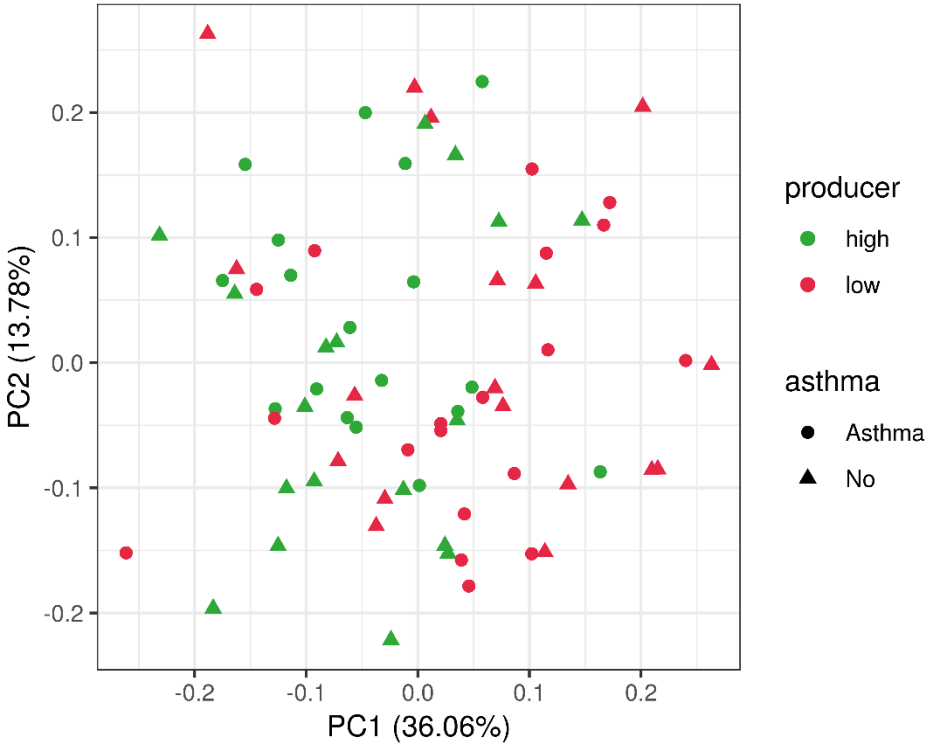


*Figure 4:4 PCA analysis of RV16-stimulated samples.*

*Green indicates high and red indicates low IFNα producer samples. Round shapes indicate asthma and triangle shapes healthy samples.*

### 4.3.4. Comparison of TLR7/8-related gene expression in high and low IFNα producer groups

Results in Chapter 3 indicated that baseline *TLR7* and *CLEC4C* gene expression in whole blood was associated with the frequency of respiratory infections, even though there was no link between IFNα production and infection frequency. We compared the gene expression levels of *TLR7* and *CLEC4C* as well as the related receptor *TLR8* and its induced cytokines *TNF* and *IL12A* at baseline and under RV16 stimulation in PBMC samples. As expected, *TLR7* and *TLR8* receptor and *IFNA*, *TNF* and *IL12A* cytokine genes were upregulated during RV16 stimulation (Figure 4:5), regardless of IFNα producer group. Only *CLEC4C* gene expression levels remained the same at baseline and during RV16 stimulation, supporting its use as a stable pDC quantity marker, unaffected by the cell activation status.

We then assessed whether TLR7/8-related genes at baseline predict an efficient IFNα response by comparing expression between the IFN-high and IFN-low groups. *CLEC4C*, *TLR7* and *TLR8* gene expression were significantly higher in the IFN-high group, indicating that baseline TLR7/8 receptor and pDC levels predict efficient IFNα response (Figure 4:5). *IL12A* baseline gene expression was also higher in the IFN-high group but not significantly after correction for multiple testing (*p*-value threshold 0.05/4 tests = 0.013). As expected, baseline *IFNA* gene expression was undetectable in both groups.

Next, we assessed the differential expression of TLR7/8-related genes in response to the virus, comparing the two IFNα producer groups. During RV16 challenge, gene expression levels of the cytokine genes *IFNA*, *TNF* and *IL12A, TLR7* receptor gene and pDC marker *CLEC4C* gene were significantly higher in the IFN-high group. However, *TLR8* gene expression was similar in IFN-high and IFN-low groups (Figure 4:5).

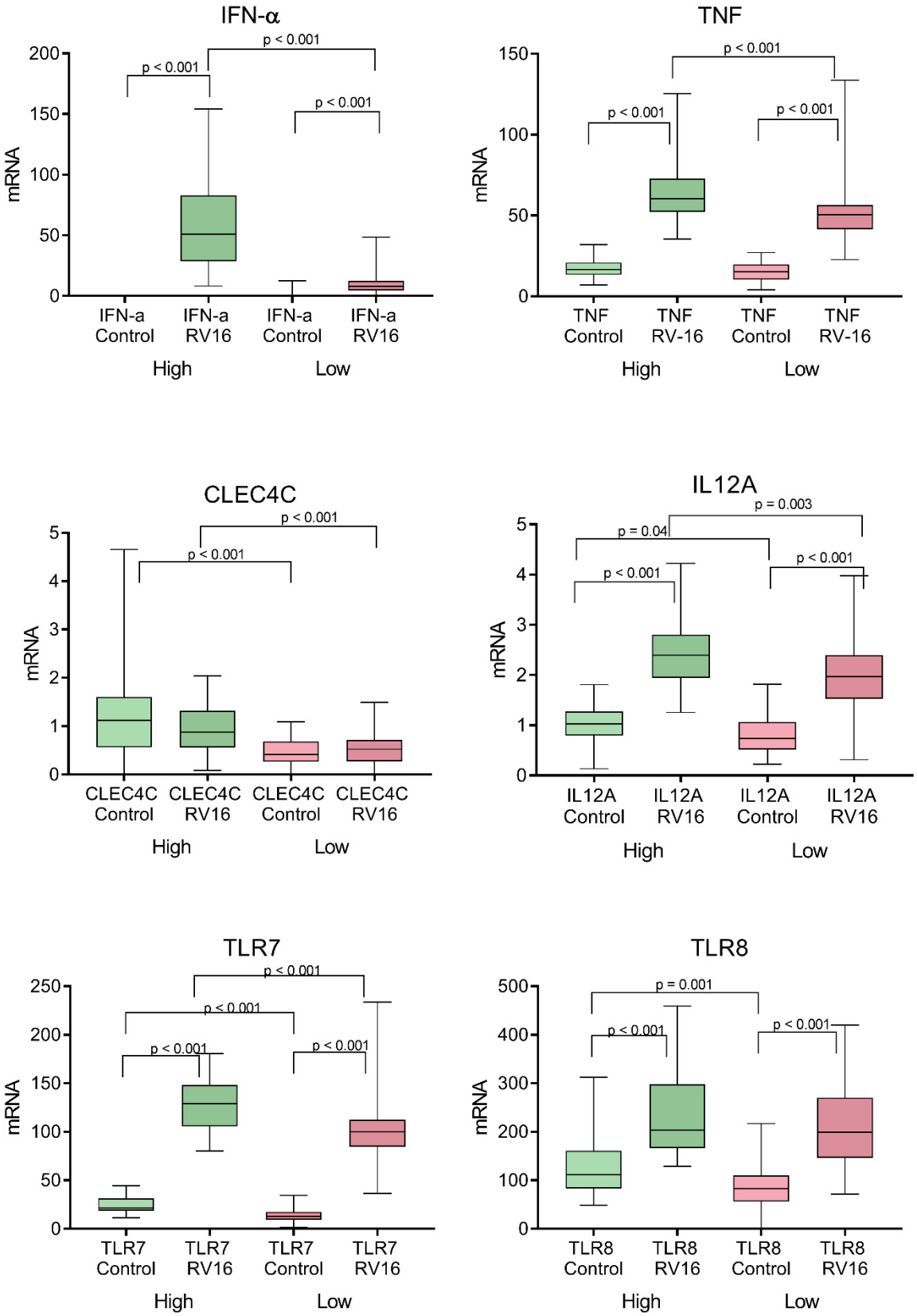


*Figure 4:5 Gene expression of TLR7/8-related immune response variables.*

*Gene expression of TLR7, TLR8, CLEC4C, the sum of IFNA genes, TNF and IL12A at baseline (control) and RV16-stimulated PBMC samples separated by IFNα producer group. p-value is shown for significant Mann-Whitney U tests.*

### 4.3.5. Other baseline gene expressions that differ between high and low IFNα producers.

Besides TLR7/8-related immunity, we wanted to determine what other biological and immunological systems at baseline explain the ability to respond to viral stimulation with robust IFNα production. Therefore, we compared the transcriptomes of the two contrasting IFNα producer groups in unstimulated PBMC samples. With a cut-off of FDR q-value < 0.05 and log fold-change > 1 of gene expression, unpaired differential gene expression analysis returned 39 upregulated genes (Table 4:2) and 10 downregulated genes (Table 4:3) in the IFN-high group. The heatmap visualisation in Figure 4:6 reveals a major subcluster of upregulated genes that are consistently upregulated in the IFN-high group and downregulated in the IFN-low group. Sample clustering with Ward hierarchical cluster analysis positions asthma samples randomly, which supports our results from PCA (Figure 4:3) that differential gene expression is not influenced by asthma status. We searched the manually annotated records in the protein knowledgebase UniProt (The UniProt Consortium 2018) and PubMed (NIH, USA) for any additional literature supporting the understanding of the functions of the gene products. Common functional groups that arose from literature are annotated for each gene in the tables below.

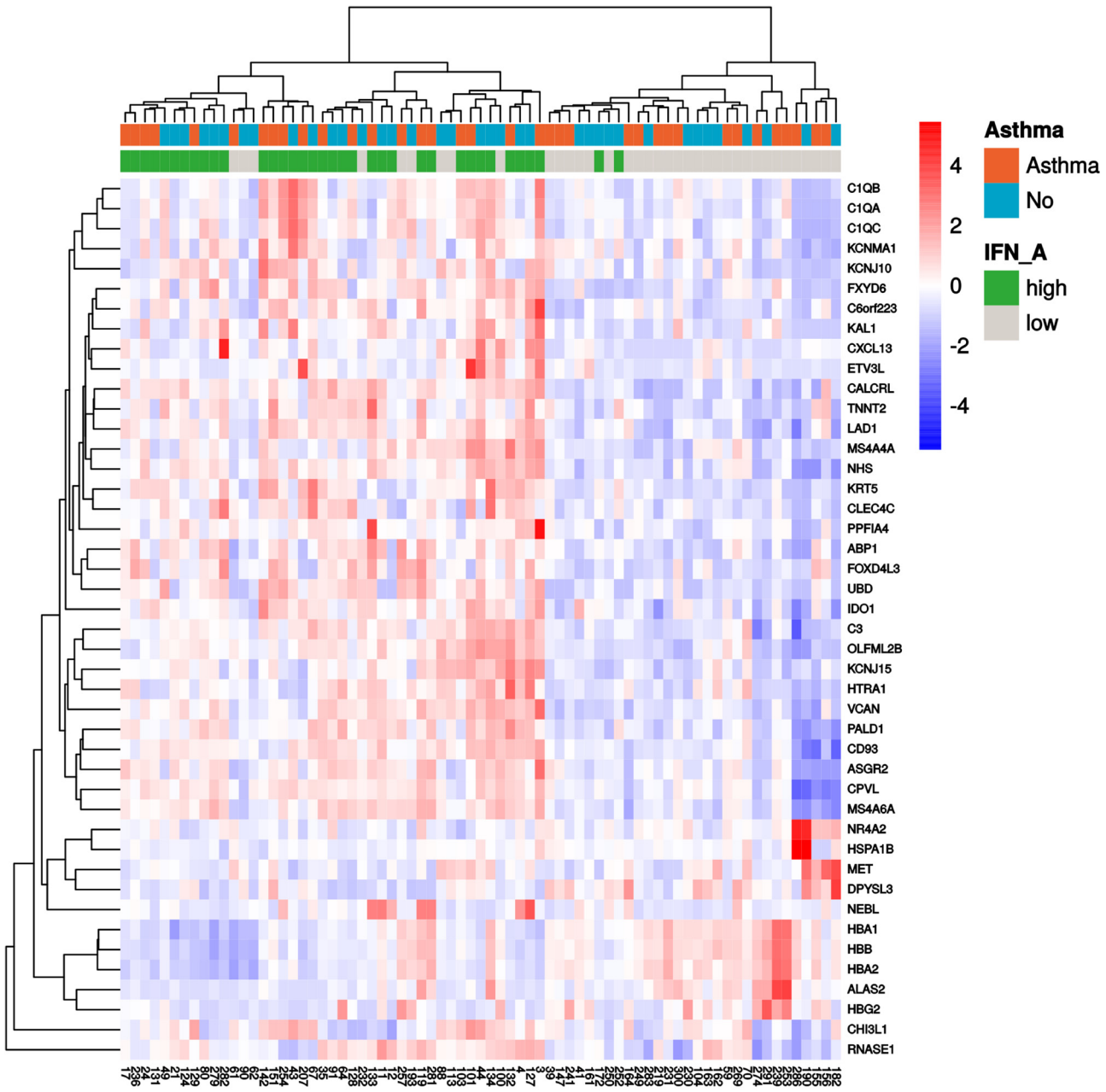


*Figure 4:6 Baseline differential gene expression.*
*Gene expression of the differentially expressed genes in unstimulated PBMC samples IFN-high group vs IFN-low group. Log2-transformed gene expression is presented as a difference from the median. Samples are hierarchically clustered with Ward method and genes with weighted pair group method with arithmetic mean. Samples are colour-coded for asthma status and IFNα producer group.*

*Table 4:2 Upregulated genes at baseline*

| Gene symbol | Gene product name | Log FC | FDR | Other | Immune function | ECM | Allergy | Lung | Ion channel | Lipid metabolism | Structural component | Transcription | Antiviral | Dendritic cells | Chemokine | Antigen presentation |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NEBL | Nebulette | 1.76 | 0.0055 | | | | | | | | * | | | | | |
| KRT5 | Keratin 5 | 1.05 | 4.32E-05 | | | | | * | | | * | | | | | |
| VCAN | Versican | 1.17 | 0.0003 | | * | * | * | * | | | | | | * | | |
| UBD | Ubiquitin D | 1.01 | 0.0009 | | * | | | | | | | | | | | |
| C1QA | Complement C1q A Chain | 1.23 | 0.0022 | | * | | | | | | | | | | | |
| C1QB | Complement C1q B Chain | 1.03 | 0.0041 | | * | | | | | | | | | | | |
| C1QC | Complement C1q C Chain | 1.71 | 0.0011 | | * | | | | | | | | | | | |
| MS4A6A | Membrane Spanning 4-Domains A6A | 1.36 | 0.0005 | | * | | | | | | | | | * | | |
| CLEC4C | C-Type Lectin Domain Family 4 Member C | 1.02 | 0.0037 | | * | | | | | | | | | * | | * |
| ANOS1 | Anosmin 1 | 1.58 | 0.0083 | | | | | | | | * | | | | | |
| ETV3L | ETS Variant 3 Like | 1.38 | 0.0321 | Vitamin D | | | | | | | | * | | | | |
| PALD1 | Phosphatase Domain Containing Paladin 1 | 1.19 | 0.0003 | | | | | * | | | | | | | | |
| CXCL13 | C-X-C Motif Chemokine Ligand 13 | 1.46 | 0.0282 | | * | | | | | | | | | | * | |
| ASGR2 | Asialoglycoprotein Receptor 2 | 1.15 | 0.0014 | | * | | | | | | | | | | | |
| NHS | NHS Actin Remodeling Regulator | 1.02 | 0.0001 | | | | | | | | * | | | | | |
| PPFIA4 | PTPRF Interacting Protein Alpha 4 | 1.31 | 0.0153 | | | * | | | | | * | | | | | |
| RNASE1 | Ribonuclease A Family Member 1, Pancreatic | 1.04 | 0.0180 | | * | | | | | | | | * | | | |
| HTRA1 | HtrA Serine Peptidase 1 | 1.43 | 0.0180 | | | * | | | | | | | | | | |
| C3 | Complement C3 | 1.13 | 0.0023 | | * | | | | | * | | | | | | |
| MS4A4A | Membrane Spanning 4-Domains A4A | 2.02 | 0.0044 | | * | | | | | | | | | * | | |
| C6orf223 | Chromosome 6 Open Reading Frame 223 | 1.28 | 0.0297 | | | | | | | | | | | | | |
| FXYD6 | FXYD Domain Containing Ion Transport Regulator 6 | 1.01 | 0.0180 | | * | | * | | | | | | | | | * |
| KCNJ15 | Potassium Voltage-Gated Channel Subfamily J Member 15 | 1.04 | 0.0142 | | | | | | * | | | | | | | |
| FOXD4L3 | Forkhead Box D4 Like 3 | 1.05 | 0.0178 | | | | | | | | | * | | | | |
| KCNJ10 | Potassium Voltage-Gated Channel Subfamily J Member 10 | 1.08 | 0.0126 | | | | | | * | | | | | | | |
| LAD1 | Ladinin 1 | 1.02 | 4.36E-05 | | | | | | | | * | | | | | |
| CHI3L1 | Chitinase 3 Like 1 | 1.16 | 0.0434 | | * | | * | * | | | | | | | | |
| TNNT2 | Troponin T2, Cardiac Type | 1.08 | 0.0109 | Cardiac | | | | | | | | | | | | |
| IDO1 | Indoleamine 2,3-Dioxygenase 1 | 1.16 | 0.0039 | | * | | | | | | | | | * | | |
| CD93 | CD93 Molecule | 1.16 | 0.0003 | | * | | * | | | | | | | * | | |
| CPVL | Carboxypeptidase, Vitellogenic Like | 1.08 | 0.0007 | | * | | | | | | | | | * | | * |
| AOC1 | Amine Oxidase, Copper Containing 1 | 1.20 | 0.0036 | | * | | * | | | | | | | | | |
| OLFML2B | Olfactomedin Like 2B | 1.09 | 0.0143 | | | * | | | | | | | | | | |
| CALCRL | Calcitonin Receptor Like Receptor | 1.24 | 0.0002 | | | | | | | | | | | | | |
| KCNMA1 | Potassium Calcium-Activated Channel Subfamily M Alpha 1 | 1.05 | 0.0095 | | | | | | * | | | | | | | |

*Upregulated genes in unstimulated PBMC samples in IFN-high group in contrast with IFN-low group and associated functional groups are annotated. FC = fold change, FDR = false*

*discovery rate. Underlined genes are differentially expressed also in RV16-stimulated samples.*

*Table 4:3 Downregulated genes at baseline*

| Gene symbol | Gene product name | Log FC | FDR | Immune function | Structural | Transcription | Antibacterial | Antiviral | Obesity | Inflammation | Oxygen transport | Wound healing |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HBA1 | Hemoglobin Subunit Alpha 1 | -1.92 | 0.0007 | | | | | | | | * | |
| HBA2 | Hemoglobin Subunit Alpha 2 | -1.89 | 0.0005 | | | | | | | | * | |
| HBB | Hemoglobin Subunit Beta | -1.97 | 0.0005 | | | | | | | | * | |
| PRAME | Preferentially Expressed Antigen In Melanoma | -1.87 | 0.0328 | * | | * | * | * | | | | |
| HBG2 | Hemoglobin Subunit Gamma 2 | -1.51 | 0.0022 | | | | | | | * | * | |
| ALAS2 | 5'-Aminolevulinate Synthase 2 | -1.65 | 0.0154 | | | | | | | | * | |
| DPYSL3 | Dihydropyrimidinase Like 3 | -1.75 | 0.0062 | | * | | | | | | | |
| HSPA1B | Heat Shock Protein Family A (Hsp70) Member 1B | -1.00 | 0.0031 | | | | | | | | | * |
| MET | MET Proto-Oncogene, Receptor Tyrosine Kinase | -1.07 | 0.0299 | | | | | | * | | | |
| NR4A2 | Nuclear Receptor Subfamily 4 Group A Member 2 | -1.88 | 0.0183 | | | * | | | | | | |

*Downregulated genes in unstimulated PBMC samples in IFN-high group in contrast with IFN-low group and associated functional groups are annotated. FC = fold change, FDR = false discovery rate. Underlined genes are differentially expressed also in RV16-stimulated samples.*

The genes upregulated by the IFN-high group in comparison to the IFN-low group were most often related to immune function (Table 4:2), whereas the downregulated genes were most often related to oxygen transport (Table 4:3). The immune-related genes included those associated with the complement system (*C1QA, C1QB, C1QC, C3, CD93*), antigen presentation (*CLEC4C, CPVL, RNASE1, ASGR2*), B-cell chemokine (*CXCL13*), immune regulation (*IDO1, CHI3L1, UBD, VCAN*), macrophage and pDC surface markers (*MS4A4A, MS4A6A*). Three potassium channel encoding genes were upregulated (*KCNJ10, KCNJ15, and KCNMA1*). A transcription factor gene *ETV3L* associated with vitamin D activation was upregulated in the IFN-high samples.

Pubmed searches revealed several upregulated genes that were associated with allergy [*CD93*, (Park et al. 2017); *AOC1*, (Anvari et al. 2015); *FXYD6*, (Chhiba et al. 2017)], asthma risk [*CHI3L1*, (Ober et al. 2008)] airway remodelling [*VCAN*, (Ayars et al. 2013); *KRT5*, (Ray et al. 2016)] and airway obstruction [*PALD1*, (Egana et al. 2017)]; however, the heatmap (Figure 4:6) shows no distinct pattern in asthma samples in relation to these genes.

A group of downregulated oxygen transport genes encode haemoglobin subunits (*HBA1, HBA2, HBB, HBG2)* and an enzyme involved in haem biosynthesis (*ALAS2*) (Table 4:3). The gene group forms a subcluster that is downregulated in most IFN-high samples (Figure 4:6). Four other downregulated genes have a role in wound healing (*MET, DPYSL3*), stress response (*HSPA1B*) and as inflammatory markers (*HSPA1B* and *NR4A2*).

### 4.3.6. Baseline biological pathway analysis of differentially expressed genes

Gene set enrichment analysis (GSEA) was used to search for functional enrichment within the differentially expressed gene sets. Figure 4:7 presents the gene ontology (GO) pathways associated with differentially expressed genes from unstimulated samples for the IFN-high group compared to the IFN-low group. The upregulated pathways indicate upregulation of immune function, whereas the downregulated pathways are more varied in their function. A network representation visualises shared genes in selected pathways, (Figure 4:8), demonstrating considerable gene overlap within both up- and downregulated pathways.

The list of differentially expressed genes resulted in several downregulated enriched pathways. The pathway for a response to oxidative stress was highly significant, with only a few gene members causing a strong effect. These gene members included: haemoglobin subunits (*HBA1, HBA2, HBB*) and inflammatory markers (*HSPA1B and NR4A2*). Together the downregulated gene functions and the enriched GO-pathways indicate a differential state of cellular stress between the IFNα producer groups, such that the cells from high IFNα producing individuals showed efficient downregulation of oxidative stress gene expression, whereas a subset low IFNα producing individuals exhibited high expression of oxidative stress gene expression.

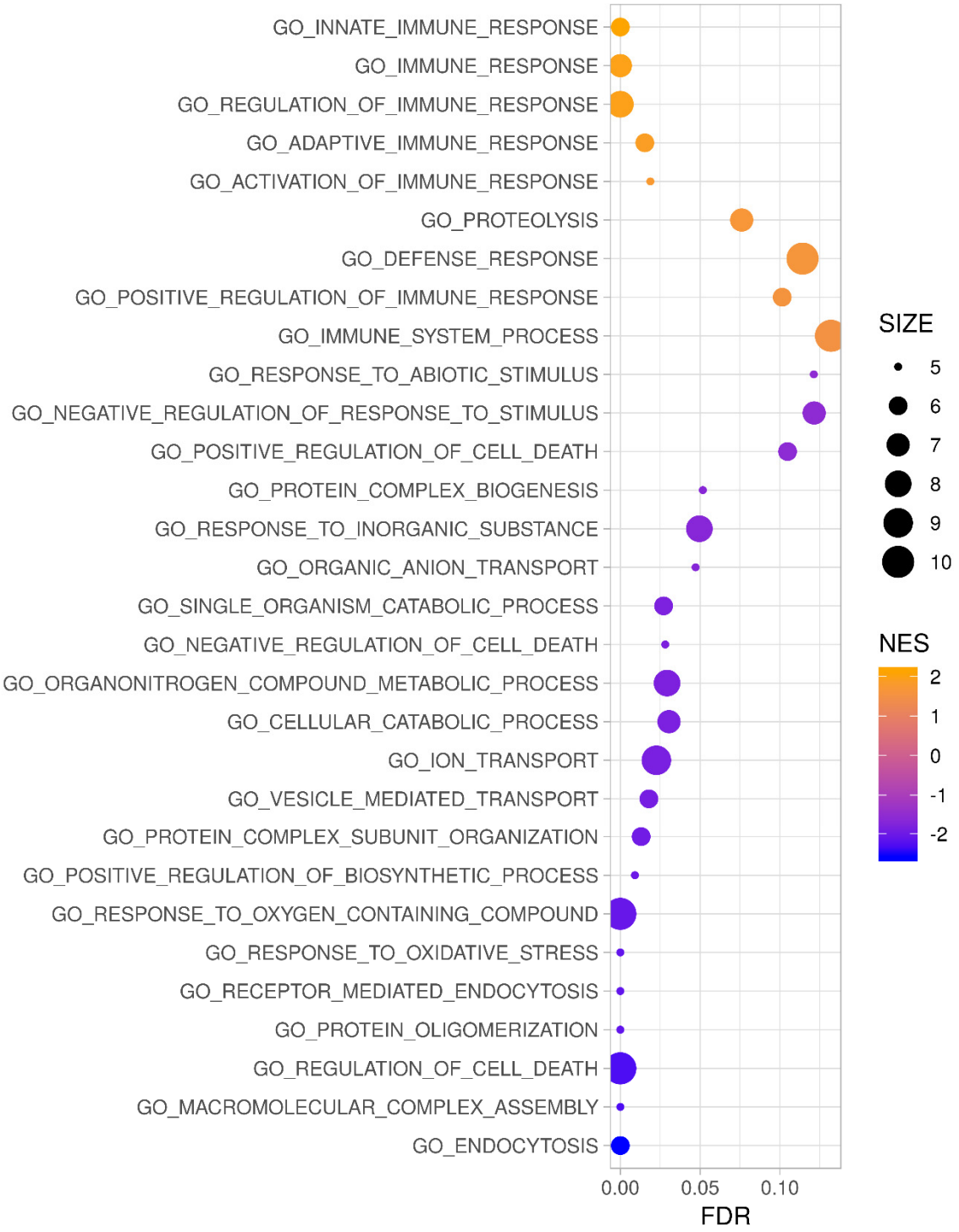


*Figure 4:7 Baseline gene ontology pathways.*
*Gene ontology pathways that are enriched in the set of differentially expressed genes in unstimulated PBMC samples of IFN-high group in contrast with IFN-low group. FDR = false discovery rate, NES = normalised effect size, size = number of genes found in the pathway. Pathways with size > 5 are included. Pathways are sorted by significance on the horizontal axis and effect on the vertical axis as indicated by the dots. Size of the dot is relevant to the number of genes found in the pathway and colour to the effect size.*

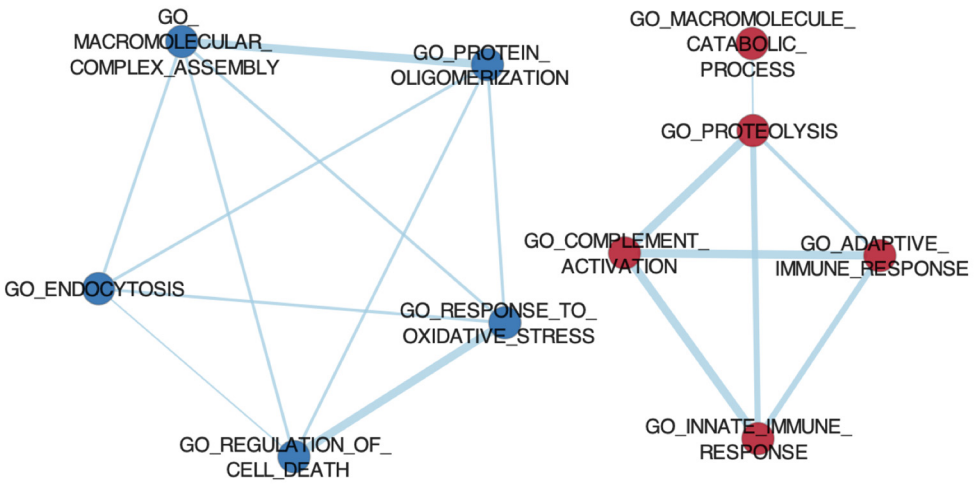


*Figure 4:8 Baseline gene ontology pathway associations.*
*Associations between selected downregulated (blue) and upregulated (red) gene ontology pathways in unstimulated PBMC samples. Pathways are enriched in the differentially expressed genes in the samples of IFN-high group in contrast IFN-low group. Pathways with least overlap in function were selected. Line thickness reflects the number of shared genes.*

### 4.3.7. RV16-stimulated gene expression and how this differs between high and low IFNα producers

To understand the variation in antiviral immune response between the IFNα producer groups, we next assessed differential gene expression during viral challenge with RV16. With a cut-off of FDR q-value < 0.05 and log fold-change > 1, unpaired differential gene expression analysis returned 55 upregulated genes (Table 4:4), and 73 downregulated genes (Table 4:5) in the IFN-high group. A heatmap graphically presents the gene expression across the samples (Figure 4:9). Similar to the clustering analysis of the unstimulated samples (Figure 4:6), the RV16-stimulated samples cluster primarily by the IFN-producer group, rather than by the presence or absence of asthma.

This heatmap reveals three distinct clusters of genes with similar expression patterns. The gene cluster in the middle contains the upregulated genes, including IFN genes. The expression of those genes is uniformly high in the IFN-high group and low in the IFN-low group.

In contrast, the downregulated genes separate into two main clusters at the top and bottom of the heatmap. The downregulated gene expression is consistently low in the IFN-high group samples but is more varied in the IFN-low group samples. The top gene cluster

contains several subclusters of genes that are upregulated by distinct sample clusters. Whereas, the bottom cluster of genes is upregulated only by one IFN-low sample cluster consisting of mostly non-asthma samples.

Some clustering with asthma and healthy samples is evident, and these sample subclusters upregulate specific gene subclusters. For example, a sample subcluster of asthmatic IFN-low samples in the far right of Figure 4:9 has a high expression of two gene clusters: *PGLYRP1, HP, TCN1, CRISP3, MMP8, CEACAM6, CEACAM8, BPI, LCN2, ALAS2* and *HBA2, HBA1, HBB, LTF*.

We searched the manually annotated records in the protein knowledgebase UniProt, and any additional literature available for the function of the differentially expressed gene products. The most common relevant functions are annotated for each gene in Table 4:4 and Table 4:5.

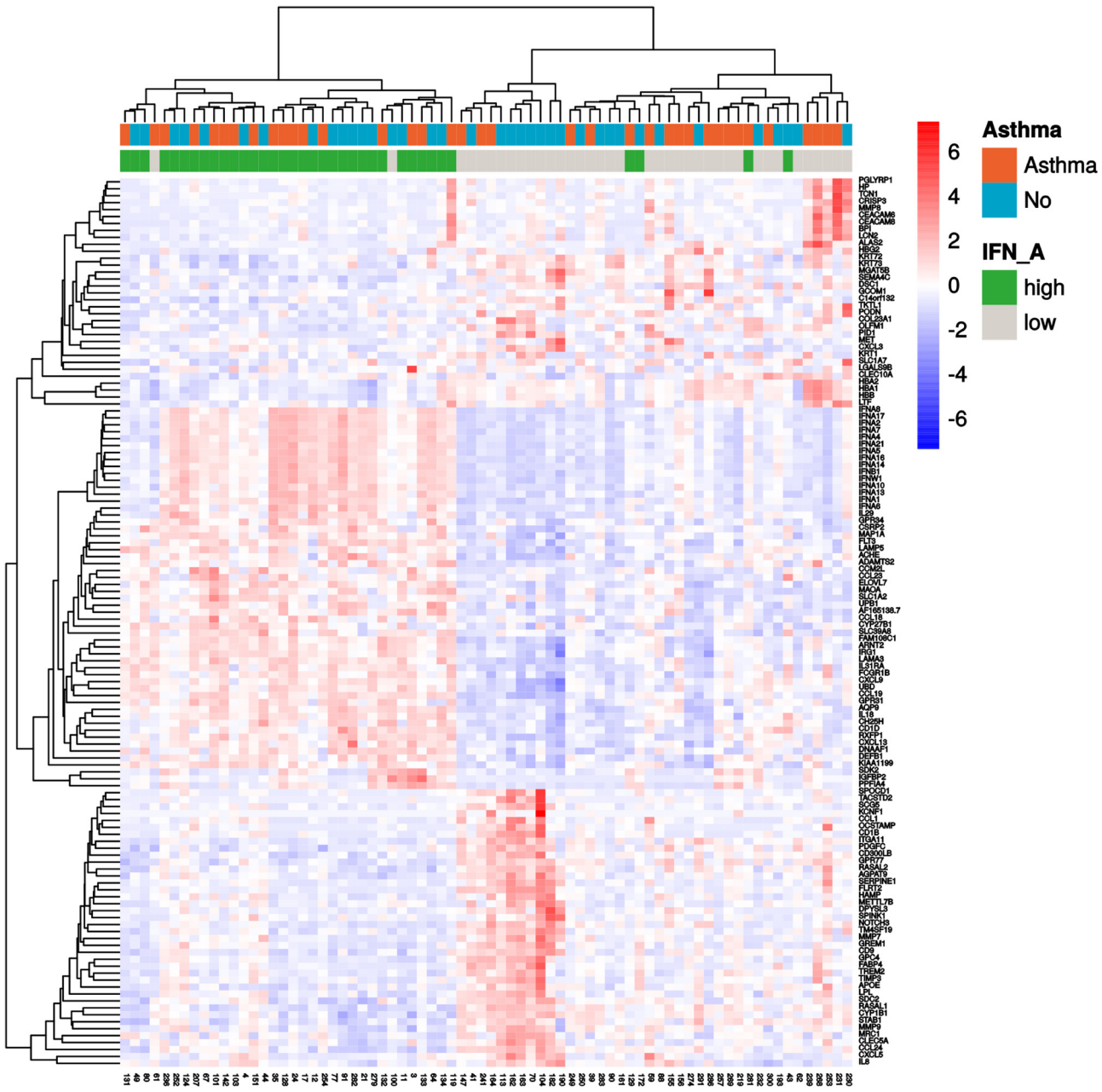


*Figure 4:9 RV16-challenged differential gene expression.*
*Gene expression of the differentially expressed genes in RV16-stimulated PBMC samples IFN-high group vs IFN-low group. Log2-transformed gene expression is presented as a difference from the median. Samples are hierarchically clustered with Ward method and genes with weighted pair group method with arithmetic mean. Samples are colour-coded for asthma status and IFNα producer group.*

*Table 4:4 Upregulated genes during RV16 challenge*

| Gene symbol | Gene product name | Log FC | FDR | Other | Immune function | ECM | Allergy | Lung | Ion channel | Lipid metabolism | Structural | Transcription | Antibacterial | Antiviral | Chemokine | Antigen presentation | Cell growth and differentiation | Migration |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IFNA8 | Interferon Alpha 8 | 2.67 | 3.32E-11 | | * | | | | | | | | | * | | | | |
| IFNA21 | Interferon Alpha 21 | 2.64 | 1.74E-10 | | * | | | | | | | | | * | | | | |
| IFNA4 | Interferon Alpha 4 | 2.62 | 1.74E-10 | | * | | | | | | | | | * | | | | |
| IFNA1 | Interferon Alpha 1 | 2.52 | 1.74E-10 | | * | | | | | | | | | * | | | | |
| IFNA16 | Interferon Alpha 16 | 2.42 | 1.79E-10 | | * | | | | | | | | | * | | | | |
| IFNA5 | Interferon Alpha 5 | 2.38 | 1.74E-10 | | * | | | | | | | | | * | | | | |
| IFNA7 | Interferon Alpha 7 | 2.26 | 6.39E-10 | | * | | | | | | | | | * | | | | |
| IFNA17 | Interferon Alpha 17 | 2.48 | 5.45E-10 | | * | | | | | | | | | * | | | | |
| IFNW1 | Interferon Omega 1 | 1.40 | 1.74E-10 | | * | | | | | | | | | * | | | | |
| IFNA2 | Interferon Alpha 2 | 2.37 | 4.01E-10 | | * | | | | | | | | | * | | | | |
| IFNA14 | Interferon Alpha 14 | 2.39 | 1.17E-09 | | * | | | | | | | | | * | | | | |
| IFNA10 | Interferon Alpha 10 | 2.40 | 2.57E-09 | | * | | | | | | | | | * | | | | |
| IFNB1 | Interferon Beta 1 | 2.33 | 1.74E-10 | | * | | | | | | | | | * | | | | |
| IFNA13 | Interferon Alpha 13 | 2.30 | 2.17E-08 | | * | | | | | | | | | * | | | | |
| IFNA6 | Interferon Alpha 6 | 2.21 | 6.33E-06 | | * | | | | | | | | | * | | | | |
| CXCL13 | C-X-C Motif Chemokine Ligand 13 | 1.87 | 2.01E-05 | | | | | | | | | | | | * | | | |
| RXFP1 | Relaxin Family Peptide Receptor 1 | 1.53 | 2.17E-08 | | | | | * | | | | | | | | | | |
| LAMA3 | Laminin Subunit Alpha 3 | 1.69 | 1.58E-07 | | | * | | * | | | | | | | | | | |
| CCL19 | C-C Motif Chemokine Ligand 19 | 1.07 | 4.87E-08 | | * | | | | | | | | | | * | | | |
| AF165138.7 | | 1.574 | 1.41 | | | | | | | | | | | | | | | |
| PPFIA4 | PTPRF Interacting Protein Alpha 4 | 1.74 | 0.0128 | | | * | | | | | | | | | | | | |
| IL18 | Interleukin 18 | 1.07 | 2.17E-08 | | * | | | | | | | | | | | | | |
| CEMIP | Cell Migration Inducing Hyaluronidase 1 | 1.17 | 8.55E-05 | | | | * | | | | | | | | | | | |
| CCL18 | C-C Motif Chemokine Ligand 18 | 1.20 | 4.36E-05 | | * | | | | | | | | | | * | | | |
| IFNL1 | Interferon Lambda 1 | 1.57 | 0.0005 | | * | | | | | | | | | * | | | | |
| AQP9 | Aquaporin 9 | 1.31 | 1.32E-07 | | | | | | * | | | | * | | | | | |
| ELOVL7 | ELOVL Fatty Acid Elongase 7 | 1.35 | 3.57E-10 | | | | | | | * | | | | | | | | |
| DEFB1 | Defensin Beta 1 | 2.01 | 0.0001 | | * | | | | | | | | * | | * | | | * |
| DNAAF1 | Dynein Axonemal Assembly Factor 1 | 1.18 | 2.77E-06 | | | | | | | | * | | | | | | | |
| IGFBP2 | Insulin Like Growth Factor Binding Protein 2 | 1.24 | 0.0484 | | | | | | | | | | | | | | * | |
| ACOD1 | Aconitate Decarboxylase 1 | 1.27 | 2.10E-06 | | * | | | | | | | | * | * | | | | |
| SLC39A8 | Solute Carrier Family 39 Member 8 | 1.30 | 1.72E-05 | | | | | | * | | | | | | | | | |
| GPR31 | G Protein-Coupled Receptor 31 | 1.98 | 0.0005 | unknown | | | | | | | | | | | | | | |
| ARNT2 | Aryl Hydrocarbon Receptor Nuclear Translocator 2 | 1.20 | 1.51E-05 | | | | | | | | | * | | | | | | |
| CCL23 | C-C Motif Chemokine Ligand 23 | 1.46 | 0.0078 | | * | | | | | | | | | | * | | | |
| CH25H | Cholesterol 25-Hydroxylase | 1.49 | 0.0006 | | * | | | | | | | | | * | | | | |
| CD1D | CD1d Molecule | 1.21 | 0.0009 | | * | | | | | | | | | | | * | | |

| Gene symbol | Gene product name | Log FC | FDR | Other | Immune function | ECM | Allergy | Lung | Ion channel | Lipid metabolism | Structural | Transcription | Antibacterial | Antiviral | Chemokine | Antigen presentation | Cell growth and differentiation | Migration |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| UBD | Ubiquitin D | 1.39 | 1.29E-05 | | | | | | | | | | | | | | | |
| SDK2 | Sidekick Cell Adhesion Molecule 2 | 1.19 | 0.0347 | development | | | | | | | | | | | | | | |
| SLC1A2 | Solute Carrier Family 1 Member 2 | 1.18 | 0.0001 | | | | | | * | | | | * | | | | | |
| CYP27B1 | Cytochrome P450 Family 27 Subfamily B Member 1 | 1.14 | 3.13E-05 | Vitamin D | | | | | | | | | | | | | | |
| UPB1 | Beta-Ureidopropionase 1 | 1.42 | 1.57E-06 | | | | | | | | | | | * | | | | |
| ACHE | Acetylcholinesterase (Cartwright Blood Group) | 1.28 | 0.0002 | acetycholinesterase | | | | | | | | | | | | | | |
| IL31RA | Interleukin 31 Receptor A | 1.25 | 0.0002 | | * | | | | | | | | | | | | | |
| LAMP5 | Lysosomal Associated Membrane Protein Family Member 5 | 1.24 | 8.26E-06 | | * | | | | | | | | | | | | | |
| ABHD17C | Abhydrolase Domain Containing 17C | 1.04 | 3.84E-07 | | | | | | | * | | | | | | | | |
| CCM2L | CCM2 Like Scaffold Protein | 1.10 | 0.0077 | cardiac | | | | | | | | | | | | | | |
| CSRP2 | Cysteine And Glycine Rich Protein 2 | 1.10 | 0.0002 | | * | | | | | | | | | | | | * | |
| MAOA | Monoamine Oxidase A | 1.08 | 0.0018 | monoamine oxidiser | | | | | | | | | | | | | | |
| GPR34 | G Protein-Coupled Receptor 34 | 1.03 | 0.0020 | | * | | | | | | | | | | | | | |
| LGALS9B | Galectin 9B | 1.15 | 0.0485 | binding protein | | | | | | | | | | | | | | |
| CXCL9 | C-X-C Motif Chemokine Ligand 9 | 1.26 | 0.0022 | | * | | | | | | | | | | | | * | * |
| FLT3 | Fms Related Tyrosine Kinase 3 | 1.54 | 1.11E-07 | | * | | | | | | | | | | | | * | |
| MAP1A | Microtubule Associated Protein 1A | 1.22 | 3.02E-07 | | | | | | | | * | | | | | | | |
| FCGR1B | Fc Fragment Of IgG Receptor Ib | 1.32 | 0.0016 | | * | | | | | | | | | | | * | | |
| ADAMTS2 | ADAM Metallopeptidase With Thrombospondin Type 1 Motif 2 | 1.09 | 0.0056 | | | * | | | | | | | | | | | | |

*Upregulated genes in RV16-stimulated PBMC samples in IFN-high group in contrast with IFN-low group and associated functional groups are annotated. FC = fold change, FDR = false discovery rate. Underlined genes are also differentially expressed in unstimulated samples.*

*Table 4:5 Downregulated genes during RV16 challenge*

| Gene symbol | Gene product name | Log FC | FDR | Other | Immune | ECM | Lung | Transport channel | Lipid metabolism | Structural | Transcription | Antibacterial | Antiviral | Obesity | Iron/Oxygen transport | Cell growth | Chemokine | Antigen presentation | Migration |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FABP4 | Fatty Acid Binding Protein 4 | -2.65 | 0.0009 | | | | | | * | | | | | | | | | | |

| Gene symbol | Gene product name | Log FC | FDR | Other | Immune | ECM | Lung | Transport channel | Lipid metabolism | Structural | Transcription | Antibacterial | Antiviral | Obesity | Iron/Oxygen transport | Cell growth | Chemokine | Antigen presentation | Migration |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CXCL5 | C-X-C Motif Chemokine Ligand 5 | -2.50 | 0.0012 | | * | | | | | | | | | | | | * | | |
| HBB | Hemoglobin Subunit Beta | -2.29 | 1.34E-05 | | | | | | | | | | | | * | | | | |
| HBA1 | Hemoglobin Subunit Alpha 1 | -2.25 | 2.35E-05 | | | | | | | | | | | | * | | | | |
| ALAS2 | 5'-Aminolevulinate Synthase 2 | -2.22 | 0.0040 | | | | | | | | | | | | * | | | | |
| NOTCH3 | Notch 3 | -2.20 | 5.37E-06 | | | | | | | | | | | | | * | | | |
| CCL24 | C-C Motif Chemokine Ligand 24 | -2.17 | 3.00E-05 | | * | | | | | | | | | | | | * | | |
| FLRT2 | Fibronectin Leucine Rich Transmembrane Protein 2 | -2.12 | 3.07E-05 | | | | | | | * | | | | | | | | | * |
| DPYSL3 | Dihydropyrimidinase Like 3 | -2.07 | 0.0017 | | | | | | | * | | | | | | | | | * |
| HBA2 | Hemoglobin Subunit Alpha 2 | -2.04 | 4.77E-05 | | | | | | | | | | | | * | | | | |
| GPC4 | Glypican 4 | -2.01 | 0.0011 | | | * | | | | | | | | | | * | | | |
| SERPINE1 | Serpin Family E Member 1 | -1.97 | 0.0004 | | | | | | | * | | | | | | | | | * |
| PID1 | Phosphotyrosine Interaction Domain Containing 1 | -1.96 | 0.0005 | | | | | | * | | | | | | | | | | |
| MMP7 | Matrix Metallopeptidase 7 | -1.93 | 0.0034 | | | * | | | | | | | | | | | | | |
| MMP8 | Matrix Metallopeptidase 8 | -1.92 | 0.0060 | | | * | | | | | | | | | | | | | |
| GCOM1 | GRINL1A Complex Locus 1 | -1.89 | 0.0050 | | | | | | | | * | | | | | | | | |
| ITGA11 | Integrin Subunit Alpha 11 | -1.87 | 1.32E-05 | | | * | | | | * | | | | | | | | | |
| RASAL1 | RAS Protein Activator Like 1 | -1.87 | 2.57E-09 | Ras-cyclic AMP pathway | | | | | | | | | | | | | | | |
| LPL | Lipoprotein Lipase | -1.78 | 0.0012 | | | | | | * | | | | | | | | | | |
| LTF | Lactotransferrin | -1.73 | 0.021 | | | | | | | | | * | | | | | | | |
| TACSTD2 | Tumor Associated Calcium Signal Transducer 2 | -1.70 | 0.0041 | | * | | | | | | | | | | | | | | |
| CYP1B1 | Cytochrome P450 Family 1 Subfamily B Member 1 | -1.68 | 1.11E-07 | | | | | | | | | | | | * | | | | |
| OCSTAMP | Osteoclast Stimulatory Transmembrane Protein | -1.64 | 0.0039 | | | | | | | | | | | | | * | | | |
| TM4SF19 | Transmembrane 4 L Six Family Member 19 | -1.60 | 0.0006 | | * | | | | | | | | | | | | | | |
| BPI | Bactericidal Permeability Increasing Protein | -1.59 | 0.021 | | * | | | | | | | * | | | | | | | |
| CD300LB | CD300 Molecule Like Family Member B | -1.59 | 4.59E-05 | | * | | | | * | | | | | | | | | | |
| CLEC5A | C-Type Lectin Domain Containing 5A | -1.59 | 0.0039 | | * | | | | | | | | | | | | | | |
| MGAT5B | Alpha-1,6-Mannosylglycoprotein 6-Beta-N-Acetylglucosaminyltransferase B | -1.55 | 0.0007 | | * | | | | | | | | | | | | | | |
| LCN2 | Lipocalin 2 | -1.54 | 0.033 | | | | | | | | | * | | | * | | | | |
| STAB1 | Stabilin 1 | -1.51 | 3.84E-07 | | | | | | * | | | * | | | | | | | |

| Gene symbol | Gene product name | Log FC | FDR | Other | Immune | ECM | Lung | Transport channel | Lipid metabolism | Structural | Transcription | Antibacterial | Antiviral | Obesity | Iron/Oxygen transport | Cell growth | Chemokine | Antigen presentation | Migration |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GREM1 | Gremlin 1, DAN Family BMP Antagonist | -1.50 | 0.011 | | * | * | * | | | | | | | | | | | | * |
| MRC1 | Mannose Receptor C-Type 1 | -1.46 | 0.0084 | | * | | | | | | | | | | | | | * | |
| CXCL8 | C-X-C Motif Chemokine Ligand 8 | -1.46 | 0.0042 | | * | | | | | | | | | | | | * | | |
| HBG2 | Hemoglobin Subunit Gamma 2 | -1.44 | 0.0032 | | | | | | | | | | | | * | | | | |
| CD1B | CD1b Molecule | -1.44 | 0.014 | | * | | | | | | | | | | | | | * | |
| CRISP3 | Cysteine Rich Secretory Protein 3 | -1.38 | 0.016 | | | | | | | | | | * | | | | | | |
| HP | Haptoglobin | -1.38 | 0.014 | | | | | | | | | * | | | * | | | | |
| PGLYRP1 | Peptidoglycan Recognition Protein 1 | -1.38 | 0.024 | | | | | | | | | * | | | | | | | |
| CD9 | CD9 Molecule | -1.38 | 0.0003 | | * | | | | | | | | | | | | | | |
| APOE | Apolipoprotein E | -1.38 | 0.0043 | | | | | | * | | | | | | | | | | |
| CLEC10A | C-Type Lectin Domain Containing 10A | -1.37 | 0.0055 | | * | | | | | | | | | | | | | | |
| COL23A1 | Collagen Type XXIII Alpha 1 Chain | -1.36 | 0.0035 | | | * | | | | | | | | | | | | | |
| CEACAM8 | Carcinoembryonic Antigen Related Cell Adhesion Molecule 8 | -1.36 | 0.049 | | | | | | | * | | | | | | | | | |
| SDC2 | Syndecan 2 | -1.34 | 0.0007 | | | | | | | * | | | | | | | | | |
| TKTL1 | Transketolase Like 1 | -1.31 | 0.0027 | | | | | | | | | | | | | * | | | |
| SPOCD1 | SPOC Domain Containing 1 | -1.30 | 0.027 | | | | | | | | * | | | | | * | | | |
| TCN1 | Transcobalamin 1 | -1.29 | 0.039 | vitamin B12 | | | | | | | | | | | | | | | |
| CEACAM6 | Carcinoembryonic Antigen Related Cell Adhesion Molecule 6 | -1.29 | 0.037 | | | | | | | * | | | | | | | | | |
| OLFM1 | Olfactomedin 1 | -1.28 | 0.0034 | | | | | | | * | | | | | | | | | |
| KRT72 | Keratin 72 | -1.27 | 0.0005 | | | * | | | | | | | | | | | | | |
| SCG5 | Secretogranin V | -1.23 | 0.018 | chaperone | | | | | | | | | * | | | | | | |
| METTL7B | Methyltransferase Like 7B | -1.20 | 0.017 | methyltransferase | | | | | | | | | | | | | | | |
| SPINK1 | Serine Peptidase Inhibitor, Kazal Type 1 | -1.18 | 0.038 | | | * | | | | | | | | | | | | | |
| TIMP3 | TIMP Metallopeptidase Inhibitor 3 | -1.18 | 0.031 | | | * | | | | | | | | | | | | | |
| KCNF1 | Potassium Voltage-Gated Channel Modifier Subfamily F Member 1 | -1.17 | 0.046 | | | | | * | | | | | | | | | | | |
| MET | MET Proto-Oncogene, Receptor Tyrosine Kinase | -1.15 | 0.036 | | | | | | | | | | | | | * | | | |
| RASAL2 | RAS Protein Activator Like 2 | -1.14 | 6.72E-05 | | | | | | | | | | | | | * | | | |
| HAMP | Hepcidin Antimicrobial Peptide | -1.12 | 0.011 | | | | | | | | | * | | | * | | | | |
| CCL1 | C-C Motif Chemokine Ligand 1 | -1.12 | 0.044 | | * | | | | | | | | | | | | * | | |

| Gene symbol | Gene product name | Log FC | FDR | Other | Immune | ECM | Lung | Transport channel | Lipid metabolism | Structural | Transcription | Antibacterial | Antiviral | Obesity | Iron/Oxygen transport | Cell growth | Chemokine | Antigen presentation | Migration |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PODN | Podocan | -1.12 | 0.041 | | | | | | | | | | | | | * | | | * |
| KRT73 | Keratin 73 | -1.11 | 0.0008 | | | * | | | | | | | | | | | | | |
| C5AR2 | Complement Component 5a Receptor 2 | -1.11 | 3.84E-07 | | * | | | | | | | | | | | | * | | |
| SLC1A7 | Solute Carrier Family 1 Member 7 | -1.11 | 0.037 | | | | | * | | | | | | | | | | | |
| TREM2 | Triggering Receptor Expressed On Myeloid Cells 2 | -1.09 | 0.011 | | * | | | | | | | | | | | | | | |
| MMP9 | Matrix Metallopeptidase 9 | -1.07 | 0.0006 | | | * | | | | | | | | | | | | | * |
| DSC1 | Desmocollin 1 | -1.07 | 0.0011 | | | | | | | * | | | | | | | | | |
| CXCL3 | C-X-C Motif Chemokine Ligand 3 | -1.07 | 0.032 | | * | | | | | | | | | | | | * | | |
| KRT1 | Keratin 1 | -1.06 | 0.036 | | * | | | | | | | | | | | | | | |
| GPAT3 | Glycerol-3-Phosphate Acyltransferase 3 | -1.06 | 0.0017 | | | | | | * | | | | | | | | | | |
| PDGFC | Platelet Derived Growth Factor C | -1.04 | 0.0005 | | | | | | | * | | | | | | | | | * |
| SEMA4C | Semaphorin 4C | -1.04 | 4.61E-06 | | | | | | | * | | | | | | | | | * |
| C14orf132 | Chromosome 14 Open Reading Frame 132 | -1.01 | 0.0004 | unknown | | | | | | | | | | | | | | | |

*Downregulated genes in RV16-stimulated PBMC samples in IFN-high group in contrast with IFN-low group and associated functional groups are annotated. FC = fold change, FDR = false discovery rate. Underlined genes are also differentially expressed in unstimulated samples.*

Table 4:4 shows gene expression of the thirteen *IFNA* subtypes. As expected, *IFNB1*, *IFNL1* and *IFNW1* were increased in the IFN-high samples. Including those genes, 33 out of 56 upregulated genes are related to immune function, and two have supporting roles in the antiviral immune response: breakdown of viral lipid membrane [*ELOVL7*, (Purdy, Shenk & Rabinowitz 2015)] and nucleic acid metabolism (*UPB1*). Interestingly, several genes that are downregulated have antibacterial functions, including *BPI, HP, HAMP, LTF, LCN2, PGLYRP1*, and *STAB1*.

The other immune-related upregulated genes had a known cytokine (*IL18, IL31RA, CXCL9, FLT*), antibacterial [*DEFB1*; *ACOD1*; *AQP9*, (Perelman et al. 2016)], antiviral (*DEFB1*), chemokine (*CCL18, CCL19, CCL23, CXCL13*) or antigen presentation (*CD1D*, *FCGR1B*) function explaining their increased expression in antiviral immune response.

Conversely, under RV stimulation, cells from the IFN-high group showed downregulated gene products that were most often related to structural or extra-cellular matrix (ECM) function, often with opposing functions. Three matrix metalloproteinases (*MMP9, MMP7, MMP8*) were prominent: these aid in ECM breakdown; however, an opposing matrix metalloproteinase inactivator *TIMP3*, was also downregulated. *SERPINE1* and *COL23A1* are also associated with ECM organisation. The gene products of *COL23A1*, *GREM1*, *STAB1, CEACAM8, CEACAM6, DSC1, FLRT2, ITGA11*, and *TIMP3* have a role in cell-cell or cell-ECM adhesion. Similarly, *SEMA4C, PDGFC, MMP9, MMP7, MMP8, CEACAM6* upregulate cell migration, whereas *PODN, CYP1B1, SERPINE1, DPYSL3* downregulate cell migration. The gene products of *PDGFC, CEACAM6,* and *GREM1* upregulate cell proliferation, whereas PODN, CD9 downregulate cell proliferation. Also, *GPC4, MET, SDC2*, may be involved in cell proliferation.

Three genes were upregulated, and seven were downregulated in both unstimulated and RV16-stimulated samples. Upregulated *UBD* encodes ubiquitin-like protein tagging proteins for degradation and regulates TNF-mediated NFKB signalling essential in innate immunity but it may also activate DC maturation. *CXCL13* and *PPFIA4* genes were upregulated, and they encode a B-cell chemokine and focal adhesion regulator, respectively. IFN-high samples downregulated the haemoglobin subunits (*HBA1, HBA2, HBB, HBG2*) and haem biosynthesis enzyme (*ALAS2*), cytoskeleton remodelling (*DPYSL3*) genes and a gene (*MET*) encoding a transmembrane receptor involved in proliferation, scattering, morphogenesis and survival in both conditions.

### 4.3.8. RV16-stimulated biological pathway analysis of differentially expressed genes

GSEA was used to search for biologically relevant functions that are enriched in the differentially expressed genes in the RV16-challenged samples. As expected, the significantly upregulated GO-pathways relate to activation of antiviral immune responses (Figure 4:10). The downregulated genes are involved in cell proliferation, cell-cell communication and cell migration, which is reflected in the enriched pathways (GO_REGULATION_OF_ANATOMICAL_STRUCTURE_MORPHOGENESIS,

GO_NEGATIVE_REGULATION_OF_CELL_COMMUNICATION). However, the downregulated pathways were statistically less significant than the upregulated pathways.

A selection of a representative up- and downregulated pathways are presented in a network Figure 4:11. This network reveals high interconnectivity and several shared genes between the upregulated pathways and individual genes between the downregulated pathways.

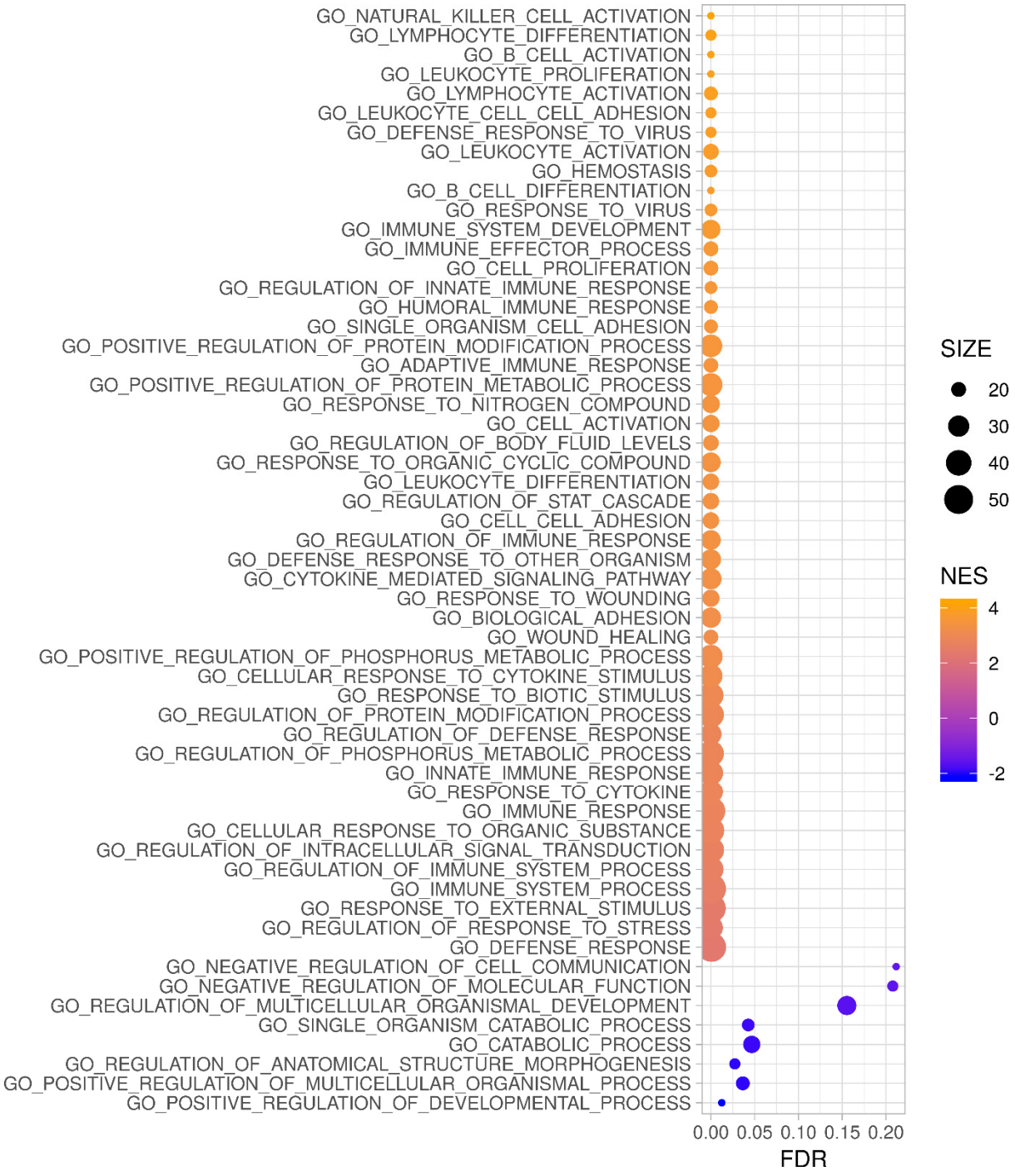


*Figure 4:10 RV16-challenged gene ontology pathways.*

*Gene ontology pathways enriched in the differentially expressed genes in RV16-stimulated PBMC samples of IFN-high group compared to IFN-low group. FDR = false discovery rate, NES = normalised effect size, size = number of genes found in the pathway. Pathways with size > 15 are included. Pathways are sorted by significance on the horizontal axis and effect on the vertical axis as indicated by the dots. Size of the dot is relevant to the number of genes found in the pathway and colour to the effect size.*

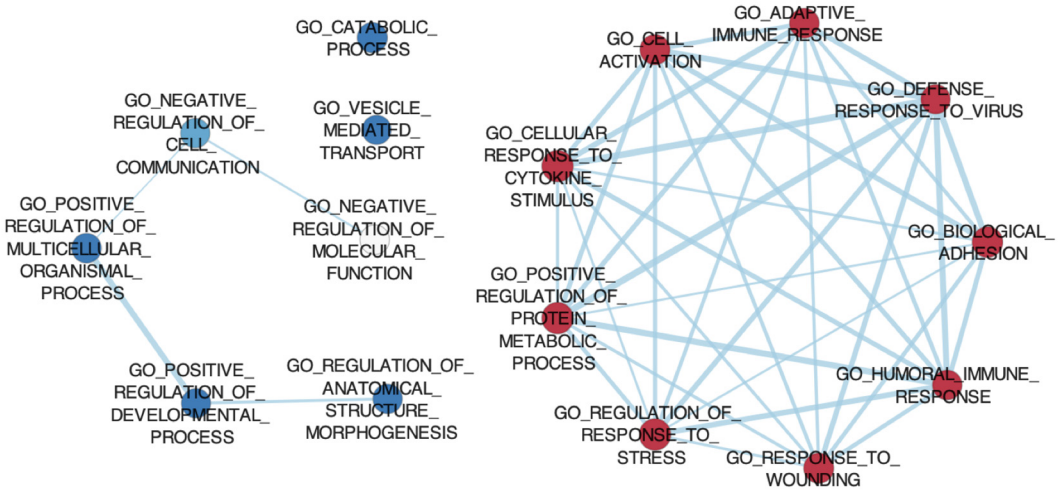


*Figure 4:11 RV16-challenged gene ontology pathway associations.*
*Associations between selected downregulated (blue) and upregulated (red) gene ontology pathways in RV16-stimulated PBMC samples. Pathways are enriched within the differentially expressed genes in the samples of IFN-high group compared to IFN-low group. Pathways were filtered for least overlap in function. Line thickness reflects the number of shared genes.*

## 4.4. Discussion

Type I IFN is vital for effective antiviral immune response. Asthma has been associated with a deficiency in type I IFN production, and despite not showing such association in Chapter 3, we observed a major degree of variability in the antiviral IFNα response between the study participants. Therefore, the first objective of this study was to identify what host and transcriptional factors predict variation in antiviral type I IFN response, and the second objective was to describe how transcriptomes vary during antiviral type I IFN response. To address these questions, we compared the transcriptomes of high and low IFNα producers at both baseline (in unstimulated PBMC) and during the antiviral response in RV16-stimulated PBMC. In relation to demographic variables, we observed that the IFN-low group had a higher median BMI, and if they had asthma, they were older relative to the IFN-high group. The IFN-high group also had a higher CLEC4C gene expression in whole blood, which reflects higher circulating pDC levels, as demonstrated in Chapter 2. Transcriptome analysis revealed differences in immune and oxidative stress-related gene expression at baseline and during the antiviral immune response, differences in gene expression related to immune response and cell proliferation, migration and attachment-related gene expression.

The current study supplements the new field of research in immune variation. At the forefront of this field is a Dutch research project, conducted as part of the Human Functional Genomics Project (HFGP), which studied immune variation in 500 healthy individuals and measured a comprehensive range of immune response variables. The outcomes of the Dutch project reported a combined effect of the environment (ter Horst et al. 2016), genetics (Schirmer et al. 2016) and microbiome (Schirmer et al. 2016) on immune variation, which was illustrated with integration of the data in a sophisticated multi-omics approach (Bakker et al. 2018). Unfortunately, those and other immune variation studies do not explain the variation in TLR7/8-mediated antiviral immune response against RV, as such, we address this gap in our study by including experiments with RV stimulated cells (Brodin et al. 2015; Lee, MN et al. 2014; Piasecka et al. 2018).

Chapter 3 demonstrated that women are more efficient IFNα responders than men, and these results concur with Berghofer et al. (2006). Piasecka et al. (2018) found that IFNα production in response to IAV was strongest in 20–29 years old healthy people, however, the effect of age on gene expression was stimulus-dependent, whereas, stronger gene

expression in females in response to antimicrobial stimuli was often shared between different stimuli. In line with their results, we observed a higher median age in the asthmatic IFN-low group; however, both healthy groups were of similar age. The associations between asthma, age and sex and their impact on antiviral immunity are complicated. We currently understand that sex hormones influence antiviral immunity (Seillet et al. 2012; Young et al. 2014) and asthma inflammation (Laffont et al. 2017), and that sex hormone levels decline with age. Consequently, in Chapter 3, we observed that age and BMI were associated with respiratory infection frequency in women only, implying a role for sex hormones in antiviral immunity.

TLR7 mediates antiviral IFNα response to RV, and initially, we investigated how the TLR7/8-related antiviral immune variables differ between the IFN-high and IFN-low groups at baseline and during the antiviral immune response. The higher baseline expression of CLEC4C, TLR7 and TLR8 gene expression in the IFN-high group is likely to indicate a better availability of the receptors and pDC hence, a higher degree of readiness to respond to viral stimulus. Besides the strong IFNα response to RV16, the IFN-high group responded more strongly to TLR8 activation with higher TNF production, indicating that the potent antiviral immune response is a predominant feature for the high responders. The differential gene expression analysis in baseline IFN-high samples, compared with IFN-low samples, further supports the theory of improved immune readiness level as the list of upregulated genes was abundant in immune-related genes and reflected in the significant immune-related GO-pathways. In further support, we showed an inverse association between baseline TLR7 gene expression in whole blood and cold frequency in Chapter 3, consistent with a level of protection from innate immune components.

Antigen-presenting dendritic cells are essential for detecting invading pathogens and activating other immune cells; therefore, they play a role in both innate and adaptive immune response. Previous work from our research group confirmed pDC as the primary and vital IFNα producer cell type (Xi et al. 2015; Xi et al. 2017), and as expected, we saw that the pDC marker *CLEC4C* was more highly expressed in the IFN-high group at baseline than in the IFN-low group, both in whole blood and PBMC (Whole blood, Table 4:1; PBMC, Figure 4:5). *CLEC4C* remained as a significant differentially expressed gene in the transcriptome analysis, indicating that pDC numbers at baseline are a key factor predicting the magnitude of the IFNα response to viral stimulation. In support of this, we discovered that another pDC

associated gene (*MS4A6A*) was highly expressed in the IFN-high group. The *CLEC4C* gene product is known to regulate both antigen presentation and type I IFN production, two functions of importance in the host response to viral infection.

Other dendritic cell-associated genes (*CD93, CPVL, IDO1, MS4A4A, VCAN*) were also upregulated in the high IFNα group at baseline supporting the close relationship between antigen-presenting cell functions and efficient IFNα response. DC are responsible for sampling the environment, presenting potential antigens, and producing cytokines to activate an antiviral immune response. One differentially expressed gene product of *ANOS1*, is thought to regulate dendrite growth, while the endosome protein encoding *ASGR2* is important for sampling the extracellular environment and *RNASE1* encoding RNAse increases the availability of viral nucleic acid, which is potentially important for DC antigen presenting ability. These findings regarding DC function are likely to complement previous studies on the role T-cells play in an efficient immune response (Bakker et al. 2018; Brodin et al. 2015). Indeed, three potassium channel encoding genes were upregulated in the high IFNα group (*KCNJ10, KCNJ15*, and *KCNMA1*) and potassium ion transport channels are thought to be essential for activating T-cells (Feske, Skolnik & Prakriya 2012).

In addition to the DC related genes, the number of upregulated genes in IFN-high samples related to the complement system was remarkable. The complement system plays a vital role in innate immunity clearing pathogens via the classical, lectin and alternative pathways. Although recently, the role of the complement system has been expanded, with a role in antiviral immunity being recognised (Hajishengallis et al. 2017). These genes are enriched in the GO-pathway for innate immune response in our study, which is consistent with the results of Bakker et al. (2018), who found that the genes with most interindividual variability in transcription were enriched in this GO-pathway (Bakker et al. 2018).

We further compared the antiviral response of the IFN-high group to the IFN-low group during RV16-stimulation. The upregulated genes reflect the stronger IFNα response in the upregulation of IFN, Th1 and antiviral immune response genes. The significantly enriched GO-pathways were also related to antiviral immune response.

Out of the 56 genes upregulated during RV16 stimulation, 10 IFN and three non-IFN genes were reported as significant cytokine response eQTL (expression quantitative trait loci) genes (Lee, MN et al. 2014). One of these genes, *IL18*, is a TLR8-induced cytokine which

activates type 1 cytokine production in natural killer (NK) cells (Gorski et al. 2006). We discovered increased *IL18* gene expression in the IFN-high response samples. The association of *IL18* with immune variation has previously been documented: an *IL18* eQTL affects IFNβ cytokine response to influenza in dendritic cells (Lee, MN et al. 2014); IL18 binding protein (IL18BP) restricts IL18 availability and inversely correlates with cytokine production (Bakker et al. 2018). The consistent findings regarding IL18 in these three studies indicate a key role for IL18 and IL18BP as immune regulators.

The high IFNα producer group downregulated more antibacterial genes than antiviral genes. Only upregulated *ACOD1* was indicated with a dual function in both antiviral and antibacterial response, whereas downregulated *BPI, HAMP, LTF* were clearly antibacterial peptides. This raises the question: Why do low IFNα producers expressed these genes during the antiviral response? One explanation could be an immune response that is unspecific to pathogen type, while high IFNα producers respond with a highly specific antiviral response. Another explanation may be related to co-infections. Although all subjects were recruited in a healthy state, and infection free for at least two weeks prior to sample donation, we cannot exclude the possibility that the low IFNα producers had a subtle underlying bacterial infection, or that these observations might be related to changes in the airway microbiome. Secondary bacterial infection post-viral infection is better understood than vice versa, but there is some evidence that asthmatic microbiomes contain pathogenic bacteria that may predispose individuals to a secondary viral infection. Such infection can lead to downregulated IFN response (Bellinghausen et al. 2016).

One of the interesting findings was the downregulation of oxidative stress-related genes in the high IFNα producers and persistent expression in a subset of low IFNα producers, and the enrichment of the corresponding GO-pathway. Cellular oxidative stress could be the result of external factors such as cigarette smoke in the lung or intrinsic factors like production of reactive oxygen species (ROS) in the context of airway inflammation. Our results reflect the fact that oxidative stress could be a factor constraining appropriate IFN production in response to a virus. Supporting evidence comes from a study showing that the oxidative stress response transcription factor NRF2 downregulates antiviral type III IFN production in epithelial cell models (Mihaylova et al. 2018). Oxidative stress is known to be present in asthma and correlates with clinical severity, which could explain the weak IFNα response to RV16 exposure in the asthmatic IFN-low samples (Sahiner et al. 2018; Wood

et al. 2005). The potential benefit of addressing oxidative stress in asthma would be reduced virus-related asthma exacerbations by promoting a strong antiviral immune response.

The significant difference in BMI was perhaps the most prominent differential host factor between the two groups. The BMI lower interquartile for the IFN-low producer groups were near the upper threshold of healthy BMI at 25, indicating that the majority of the IFN-low subjects were slightly overweight. On examination, during viral stimulation, many of the differentially expressed gene products engage in lipid metabolism and transport. The weight imbalance between the groups may explain why lipid transport (*FABP4, PID1, APOE*) and metabolism (*LPL, CD300LB, STAB1, GPAT3*) genes were downregulated in the healthy weight range, high IFNα producers, however, out of those genes at least *STAB1* is important for bacterial membrane breakdown and may be downregulated in viral infection due to its antibacterial role instead. The high IFNα producer group showed high expression of genes (*ELOVL7, ABHD17C*) that are important for viral envelope lipid metabolism. In Chapter 3, we discovered that BMI was associated with respiratory infection frequency, and the results in this chapter concur: IFNα production is associated with BMI.

The differential expression of genes related to vitamin availability requires mention. High IFNα producer group upregulated *ETV3L*, vitamin D associated transcription factor (Kariuki et al. 2016), in unstimulated samples; and *CYP27B1*, an active vitamin D form catalyser, in RV16-stimulated samples. *TCN1*, a vitamin B12 binding protein, was downregulated in RV16-stimulated samples. The expression of these genes indicates that the vitamin D availability may be necessary for a strong antiviral response. Similarly, by downregulating the B12 binding protein, high IFNα producers are making more B12 available (Nielsen et al. 2012).

The common denominator between high BMI, oxidative stress and vitamin availability is, of course, diet. Obesity is associated with systemic inflammation and oxidative stress and one explanation is poor diet (Manna & Jain 2015; Osadnik et al. 2019). Meta-analyses show that vitamin D supplementation, antioxidant flavonoid intake and probiotics decrease the incidence of respiratory infections, which supports our findings that vitamin availability and oxidative stress affect antiviral immunity (Martineau et al. 2017; Quick 2015; Somerville, Braakhuis & Hopkins 2016). A diet high in antioxidants may benefit antiviral immunity, and additionally, limited data show that it could alleviate asthma symptoms (Wood et al. 2008). Variation in gut microbiome was shown to particularly affect TNF and IFNγ production in

response to an immune stimulus (Schirmer et al. 2016). A sophisticated multi-omics analysis of immune variation discovered that a short-chain fatty acid metabolite produced by gut bacteria, and circulating high-density lipoprotein cholesterol, correlated negatively with influenza-induced, proinflammatory cytokine production (Bakker et al. 2018). As diet is known to change the microbiome, a poor diet may have an indirect effect on immunity via microbiome variation, and a direct effect from consumption of dietary antioxidants, fibre, fats and vitamins.

The main limitation of this study was the fact that it was not possible to perform RNA-Seq on all 238 participants of European ancestry. For economic reasons, we elected to focus the transcriptomic studies on a subset of these participants, i.e. those in the highest and lowest quartiles of IFNα production. Unfortunately, this led to some mismatching of the groups with regard to BMI, age and gender. As noted in Chapter 3, these variables are associated with antiviral immunity, and this could have biased the findings. A further limitation was that we analysed equal numbers of asthma and healthy samples. Asthma-related inflammation may have affected the gene expression, although it was not a significant factor in our principal component analysis and our results are in line with other studies, which included only healthy samples (Bakker et al. 2018; Lee, MN et al. 2014). Due to the restricted sample size, we chose to combine the asthma and healthy groups, an approach that was justified by the principal component and heat map analyses (Figures Figure 4:3, Figure 4:4, Figure 4:6, and Figure 4:9). The samples were selected based on IFNα production in response to RV16 stimulation for 24 hours; however, future studies could further address the transcriptomic difference between TLR7 and RV16 stimulated cells and examine additional time points. There has been limited research suggesting seasonal variation in the immune response to respiratory viruses; however, we did not account for this given that our samples were collected across all months between November 2014 and July 2016 (ter Horst et al. 2016).

Taken together, these results show that at baseline, high IFNα responders upregulated essential components of the innate immunity such as the complement system, pDC and DC genes. During the antiviral immune response, the high IFNα producers devote their transcriptome to the production of antiviral cytokines and effector proteins and downregulate the expression of genes encoding antibacterial proteins and cellular processes, such as cell proliferation and migration. The differential gene expression implies that people have varying

levels of immune system readiness that translates into a more pathogen-specific immune response. The difference between the high and low IFNα antiviral response may arise from individual host factors impacting oxidative stress, as well as vitamin D and B12 availability, as indicated by the differentially expressed genes. One of the most significant host factors associated with low IFNα production is BMI, which has been linked with oxidative stress. The genes associated with high and low IFNα production determined by this study may have implications for a better understanding of asthma, autoimmune disease and vaccine responses associated with aberrant type I IFN production.

# Chapter 5: The impact of genetic variation in the *TLR7/8* locus on gene expression, TLR7/8 function and immunity

## 5.1. Introduction

Pattern recognition receptors can be regarded as the burglar alarms of innate immunity, sensing conserved molecular patterns present in invading pathogens. Toll-like receptor (TLR)7 and its paralogue TLR8 are responsible for eliciting an immune response upon detecting either viral or bacterial single-stranded RNA (ssRNA) (Diebold et al. 2004; Gantier et al. 2010; Heil et al. 2004; Mancuso et al. 2009). TLR7 and TLR8 are predominantly expressed on immune cells: TLR7 in pDC and B-cells and TLR8 in monocytes, macrophages, myeloid dendritic cells and neutrophils (Hornung et al. 2002; Makni-Maalej et al. 2015). However, TLR7 is also expressed in structural cells such as bronchial epithelial cells (Ioannidis et al. 2013; Uehara et al. 2007) and airway smooth muscle cells (Ekman, Adner & Cardell 2011).

Both receptors are located in cellular endosomes wherefrom TLR7 employs a signalling pathway including MyD88, IRAK, and TRAF molecules to activate a transcription factor IRF7 for cytokine induction. In contrast, TLR8 shares the use of MyD88 but uses different pathways and activates CREB, NF-κB and AP1 as transcription factors (described in detail in Chapter 1: section 1.2.3). TLR7 and TLR8 induce the production of different cytokines: TLR7 induces type I interferon (IFN), whereas TLR8 induces proinflammatory cytokines such as TNF and IL12 (Gorden et al. 2005). However, the cytokine repertoire depends on the receptor stimulus (Bergstrom et al. 2015; Colak et al. 2014) and intercellular endosome trafficking (Miyake et al. 2018), or it can be replaced by secretion of antimicrobial proteins such as beta-defensin 2, as shown by Uehara et al. (2007). This divergence of TLR7 and TLR8 function suggests that these two receptors may have complementary roles in antiviral immunity, and their expression may be crossregulated (Ghosh et al. 2007; Paul et al. 2016) possibly by a mechanism of competing for Unc93B1 chaperone (Alexopoulou, Desnues & Demaria 2012).

The clearance of viruses is dependent on their efficient detection and subsequent antiviral immune response coordinated by cytokines. TLR7 and TLR8 detect ssRNA viruses such as HIV, hepatitis viruses, and most respiratory viruses and trigger type I IFN and proinflammatory cytokine production. In Chapter 3, we showed that baseline TLR7 gene expression inversely correlates with self-reported cold frequency, indicating that high levels of TLR7 protect from symptomatic infections. Although crucial for viral clearance, prolonged cytokine-induced inflammation further aggravates chronic diseases such as human

immunodeficiency virus (HIV) infection and hepatitis (Snell, McGaha & Brooks 2017), and robust TLR7/8 activation may not always be beneficial.

Besides their role in antiviral immunity, TLR7 and TLR8 modulate other immune responses. TLR7-induced type I IFN and TLR8-induced TNF and IL12 promote type 1 immunity rather than a type 2 immune response (Pritchard, Carroll, et al. 2012; Yu et al. 2015). Consequently, autoimmune diseases related to type 1 immunity, such as systemic lupus erythematosus (SLE) are commonly associated with increased TLR7 and TLR8 function (Lyn-Cook et al. 2014). Autoimmune diseases are typically characterised by reactivity to self-antigens, as well as reactivity to self-nucleic acid recognised by TLR7 and TLR8. In contrast, type 2-related atopic disease such as asthma, where type 1 immunity is downregulated, is associated with reduced function of TLR7 and TLR8 (Roponen et al. 2010) and mouse models show that stimulating TLR7 reverses asthma-like phenotype (Du, Q et al. 2009). The results in Chapter 3 concur as we detected lower baseline TLR7 gene expression levels in those with asthma than without. Moreover, there was evidence that the determinants of respiratory infection frequency varied with sex. TLR7 expression was an independent predictor of cold frequency in men, but not women, while rhinovirus-induced IFNα was higher in women than in men. Could these findings have a genetic basis?

The genes encoding TLR7 and TLR8 are located in close proximity on the X chromosome. Many single nucleotide polymorphisms (SNPs) in the *TLR7/TLR8* locus have been reported to associate with immune disease and function, providing some evidence that genetic variation in this region has functional consequences in several diseases. The most studied SNP in the region is rs179008, a functional SNP in *TLR7*. The rs179008: T allele was reported to associate compromised clearance of hepatitis C infection (Fakhir et al. 2018), as well as higher HIV load and accelerated disease progression (Oh et al. 2009). In contrast, others found that the same allele was associated with protection from viral disease, namely chronic hepatitis B (Buschow et al. 2018) and recurrent RV infections (Toivonen et al. 2017). This allele is also associated with increased asthma risk in girls (Törmänen et al. 2017) and increasing susceptibility to systemic lupus erythematosus (dos Santos et al. 2012). Small candidate-gene studies have reported weak associations between other variants in the *TLR7/TLR8* region and asthma (Genov et al. 2014; Moller-Larsen et al. 2008; Nilsson et al. 2012; Törmänen et al. 2017). For example, the *TLR8* SNP rs2407992 was reported to

associate with asthma and lung function (Genov et al. 2014; Moller-Larsen et al. 2008; Toivonen et al. 2017).

In summary, there is some evidence that SNPs in the TLR7/8 locus are associated with a viral infection and both type 1 and 2 cytokine response diseases. However, the molecular and cellular mechanisms underlying the reported associations are largely unknown. We hypothesised that such associations with immune-related diseases result from SNP effects on *TLR7* and *TLR8* expression and, consequently on the production of TLR7-induced type I IFN and TLR8-induced TNF and IL12 cytokines.

Specifically, in this chapter, we examined the association between genetic variation in the *TLR7/*8 locus and the expression of TLR7 and TLR8 receptors and downstream cytokine production in up to 238 individuals. We also examined genetic associations with the frequency of self-reported respiratory infection, and immune cell counts. The primary analysis focused on a single variant (rs850637) reported to associate with asthma risk in a recent genome-wide association study (GWAS) (Ferreira et al. 2019). For completeness, the analysis was then expanded to all common variants within 1 Mb of *TLR7/TLR8*.

## 5.2. Materials and methods

### 5.2.1. Participants and clinical sample collection

A total of 238 individuals (63% female) participated in this study, including 133 (56%) with asthma. To limit variation arising between different ethnic populations, we included only participants with confirmed European ancestry. They form a subgroup of the 301 individuals (151 with asthma and 150 control subjects) described in Chapter 3. Self-reported ancestry was confirmed against genetic data collected during genotyping described in section 5.2.3. Peripheral blood mononuclear cells (PBMC) from these individuals were cultured with media (CTRL), human rhinovirus(RV)16, TLR7 agonist (imiquimod) or TLR8 agonist (VTX-2447) for 24 h, and cytokines (TNF, IL12 and IFNα) were measured in the supernatant, as described in detail in Chapter 3. Additional outcomes included *TLR7* and *TLR8* gene expression in unstimulated PBMC and whole blood and in RV16-, TLR7-, or TLR8-stimulated PBMC, and immune cell counts.

### 5.2.2. Collection of genetic information

DNA was collected either from granulocytes isolated from whole blood or a saliva sample collected with an Oragene kit (DNA Genotek Inc., Canada) according to the manufacturer's instructions. DNA from saliva was extracted using an extraction kit from the manufacturer, whereas DNA from polymorphonuclear cells was extracted with in-house buffers using the salt precipitation protocol. DNA samples were extracted from four participants in duplicate for quality control.

### 5.2.3. Locus-specific and genome-wide SNP genotyping

A total of 57 SNPs in the *TLR7/8* region were genotyped at Agena Biosciences (Brisbane) with the MassARRAY iPLEX genotyping system. The SNPs consisted of: (1) 50 common SNPs (minor allele frequency (MAF) > 5%) within 100 kb of *TLR7/8* that were included in the Affymetrix Axiom array used by the UK Biobank, which was designed to maximise the ability to impute unmeasured common variants; (2) three known non-synonymous variants in *TLR7* and *TLR8*, all with MAF > 1%; and (3) four non-coding variants independently associated with *TLR7* expression in lymphoblastoid cell lines (Brumpton & Ferreira 2016). Four DNA samples were genotyped in duplicate for quality control. Across the 57 SNPs, the average sample call rate (the fraction of SNPs with a non-missing genotype call) was 99.7%.

In parallel, DNA samples were also genotyped with the Infinium® Global Screening Array-24 v1.0 at Human Genomics Facility in Erasmus MC, Rotterdam, The Netherlands. Six samples were included in duplicate for quality control. Variants within 2 Mb of TLR7 and TLR8 and that passed quality control filters (namely call rate > 98%, MAF > 1% and Hardy-Weinberg equilibrium *p*-value < $10^{-6}$) were merged with the 57 SNPs from the custom genotyping project. The resulting dataset was then uploaded to the Michigan Imputation Server (v1.0.4) for imputation of unmeasured variants. Imputation was based on the HRC reference panel (r1.1, 2016), using ShapeIT (v2.r790) for phasing. For the analysis reported in this chapter, we analysed imputed SNPs: (1) with a MAF > 5%; (2) within 1 Mb of the *TLR7/TLR8* region; and (3) imputed with high confidence (imputation $r^2$ of 0.6 or greater). This yielded a set of 1748 SNPs.

### 5.2.4. Gene expression analysis

The expression of *TLR7* and *TLR8* in whole blood (collected using PAXgene tubes) was analysed using relative quantitative (RT)-PCR in all 238 samples. The comparative Ct method (Schmittgen & Livak 2008) was applied using *B2M* (Beta-2-Microglobulin) and *UBC* (Polyubiquitin-C precursor) as reference genes. The RNA extraction and RT-PCR methods are described in more detail in Chapters 2 and 3 (Sections 2.2.5 and 3.2.4).

Additionally, transcription of *TLR7*, *TLR8*, *IFNA* genes, *TNF*, and *IL12A* were quantified in a subset of 74 control and 74 RV paired samples with RNA-sequencing performed as detailed in Chapter 4 methods (sections 4.2.3 and 4.2.4). The expression of 11 key IFNα genes (*IFNA4, IFNA7, IFNA10, IFNA16, IFNA17, IFNA13, IFNA2, IFNA8, IFNA1, IFNA14* and *IFNA5)* were summed for association analyses. In secondary analyses, we also extended our gene expression analysis to other genes near rs850637 (+/- 1 Mb). To include all available genes, we removed the filter for counts per million (CPM).

### 5.2.5. Access to public databases

In addition to the gene expression dataset generated as part of this study, we also used publicly available databases of expression quantitative trait loci (eQTL) to identify associations between SNP 850637 and gene expression. We considered three tissue types from the Genotype-Tissue Expression (GTEx) Project, namely whole blood (n = 369), lung (n = 383) and spleen (n = 146). The data used for the analyses described here were obtained from the GTEx Portal on 26/09/2018 (GTEx Consortium 2013)

The second external database used was the NHLBI Framingham SNP Health Association Resource (SHARe), a substudy of Framingham Heart Study (FHS) Cohort (Joehanes et al. 2017); dbGaP Study Accession: phs000342.v18.p11). We accessed this database on 20/09/2018 to search for any significant associations between rs850637 and gene expression in whole blood samples (n = 5622).

### 5.2.6. Statistical analysis

All statistical analyses were performed with R version 3.4.4 (R Core Team 2018). Variables were tested for normality, and an inverse-normal transformation applied. Hemizygous male genotypes were combined with female genotypes homozygous for the same allele for analysis. Associations were tested with linear or logistic regression, as appropriate. Linear regression tests were controlled for confounding as follows: dependent variables were adjusted for sex and age; *IFNA* mRNA count for IFNα producer group; monocyte, eosinophil, and basophil count for asthma; and lymphocyte count for body mass index (BMI). A permutation approach was used to adjust for multiple testing empirically. Specifically, for each permutation (out of 1,000 in total), we randomly shuffled all SNPs as a block (i.e. preserving the correlation pattern between SNPs) between individuals and then tested the association between all phenotypes and all SNPs. For each permutation, we retained the most significant *p*-value observed across all SNP-phenotype combinations tested (Pmin). The 50$^{th}$ most significant Pmin across the 1,000 permutations was considered to be the significance threshold required to maintain a 5% type-I error rate after accounting for multiple testing. Power calculations were performed with *minSlopeEQTL.SLR()* from the powerEQTL package or online tool at http://osse.bii.a-star.edu.sg/. Linkage disequilibrium was tested with online tool at https://ldlink.nci.nih.gov (Machiela & Chanock 2015).

## 5.3. Results

### 5.3.1. Does *TLR7* and *TLR8* gene expression differ between sexes?

TLR7 and TLR8 function differs between the sexes, with higher IFNα production reported in females than in males (Berghofer et al. 2006; Torcia et al. 2012). Whether this is due to differences in receptor expression at baseline, or in response to activation, or whether it is due to alterations in intra-cellular signalling pathways, has not been clarified. Moreover, both *TLR7* and *TLR8* can escape X-chromosome inactivation (XCI) in some individuals (Chung et al. 2006; Souyris et al. 2018). Given these observations, we first compared gene expression between males and females. Females and males had similar baseline (unstimulated) *TLR7* and *TLR8* gene expression in both PBMC and whole blood samples. After RV16 stimulation, *TLR7* gene expression was also similar between the sexes, but *TLR8* gene expression was higher in males than in females ($p$ = 0.003, Figure 5:1). The similarity in expression levels between the two groups at baseline suggests that in females, this locus does not escape XCI. In subsequent analyses, data from males and females were analysed jointly.

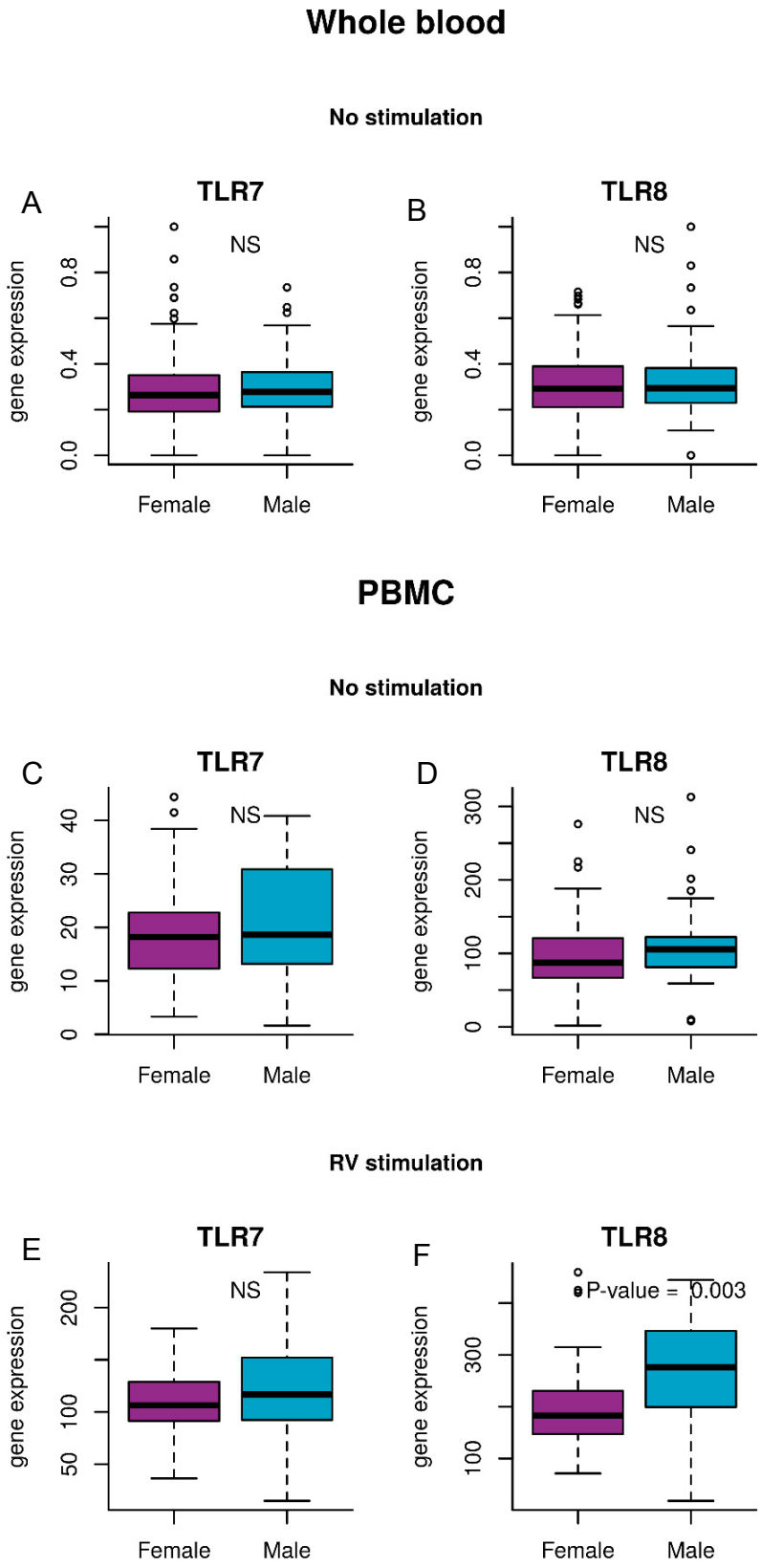


*Figure 5:1 TLR7 and TLR8 gene expression in females and males.*

*Gene expression measured in whole blood (A, B) or in PBMC (C – F) at baseline (A – D) or after RV16 stimulation (E, F). Whole blood samples n = 238 and PBMC samples n = 74. NS = not significant. p-value < 0.05 shown for t-test is considered significant.*

### 5.3.2. Association between rs850637 and the expression of nearby genes

A variant rs850637 (A/G) at position chrX:13005622 (GRCh38.p12) near *TLR7/TLR8* was reported in a recent GWAS to associate with asthma risk at the genome-wide significance level (Ferreira et al. 2019). Specifically, the rs850637:A variant was associated with a lower risk of childhood asthma (odds ratio = 0.95). To investigate the mechanisms that might underlie this association, the association of this SNP with the expression of *TLR7, TLR8* and 23 additional genes located nearby (+/- 1 Mb) was studied (Table 5:1).

*Table 5:1 Genes near rs850637*

| Gene Symbol | Start | End | Strand | Ensembl Gene ID | Gene type | Description |
|---|---|---|---|---|---|---|
| **RF00019** | 120548234 | 120549264 | - | ENSG00000206792.1 | Misc. RNA | Y_RNA (from geneSymbol) |
| **FRMPD4** | 121384654 | 127245234 | + | ENSG00000169933.12 | Protein coding | Homo sapiens FERM and PDZ domain containing 4 (FRMPD4), mRNA. (from RefSeq NM_014728) |
| **AC002981.1** | 121483174 | 121484014 | - | ENSG00000252111.1 | miRNA | AC002981.1 (from geneSymbol) |
| **RF00020** | 121491254 | 121492384 | + | ENSG00000206795.1 | Misc. RNA | Y_RNA (from geneSymbol) |
| **FRMPD4-AS1** | 123731664 | 123755134 | - | ENSG00000223487.1 | antisense | FRMPD4 antisense RNA 1 (from HGNC FRMPD4-AS1) |
| **RN7SKP290** | 126140024 | 126141974 | - | ENSG00000271814.1 | Misc. RNA | RNA, 7SK small nuclear pseudogene 290 (from HGNC RN7SKP290) |
| **PRPS2** | 127913694 | 128244224 | + | ENSG00000101911.12 | Protein coding | Homo sapiens phosphoribosyl pyrophosphate synthetase 2 (PRPS2), transcript variant 2, mRNA. (from RefSeq NM_002765) |
| **TLR7** | 128670824 | 128900384 | + | ENSG00000196664.4 | Protein coding | Homo sapiens toll like receptor 7 (TLR7), mRNA. (from RefSeq NM_016562) |
| **TLR8-AS1** | 129028164 | 129083334 | - | ENSG00000233338.1 | antisense | TLR8 antisense RNA 1 (from HGNC TLR8-AS1) |
| **TLR8** | 129066384 | 129222024 | + | ENSG00000101916.11 | Protein coding | Homo sapiens toll like receptor 8 (TLR8), transcript variant 1, mRNA. (from RefSeq NM_016610) |
| **TMSB4X** | 129751074 | 129772274 | + | ENSG00000205542.10 | Protein coding | Homo sapiens thymosin beta 4, X-linked (TMSB4X), mRNA. (from RefSeq NM_021109) |
| **FAM9C** | 130356324 | 130446814 | - | ENSG00000187268.11 | Protein coding | Homo sapiens family with sequence similarity 9 member C (FAM9C), mRNA. (from RefSeq NM_174901) |
| **RP11-791M20.1/ AC079171.1** | 130936594 | 130945734 | + | ENSG00000261030.1 | lincRNA | Homo sapiens mRNA full length insert cDNA clone EUROIMAGE 712308. (from mRNA AL079309) |
| **GS1-600G8.5** | 132660474 | 133033924 | - | ENSG00000235385.1 | lincRNA | GS1-600G8.5 (from geneSymbol) |
| **GS1-600G8.3** | 133106514 | 133199334 | + | ENSG00000231216.1 | antisense | Homo sapiens unknown transcript (GS1-600G8.3), long non-coding RNA. (from RefSeq NR_046087) |
| **ATXN3L** | 133182354 | 133199524 | - | ENSG00000123594.4 | Protein coding | Homo sapiens ataxin 3 like (ATXN3L), mRNA. (from RefSeq NM_001135995) |
| **LINC01203/ RP11-142G7.2** | 133363804 | 133736344 | + | ENSG00000226985.5 | lincRNA | long intergenic non-protein coding RNA 1203 (from HGNC LINC01203) |
| **RP11-1L9.1** | 133771874 | 134030194 | + | ENSG00000226434.1 | lincRNA | RP11-1L9.1 (from geneSymbol) |
| **EGFL6** | 135699604 | 136335744 | + | ENSG00000198759.11 | Protein coding | Homo sapiens EGF like domain multiple 6 (EGFL6), transcript variant 1, mRNA. (from RefSeq NM_015507) |
| **RN7SKP20** | 135966235 | 135966548 | + | ENSG00000199622.1 | Misc. RNA | RNA, 7SK small nuclear pseudogene 20 (from HGNC RN7SKP20) |

| Gene Symbol | Start | End | Strand | Ensembl Gene ID | Gene type | Description |
|---|---|---|---|---|---|---|
| **TCEANC** | 136596686 | 136635564 | + | ENSG00000176896.8 | Protein coding | Homo sapiens transcription elongation factor A N-terminal and central domain containing (TCEANC), transcript variant 1, mRNA. (from RefSeq NM_152634) |
| **RAB9A** | 136891240 | 137105060 | + | ENSG00000123595.6 | Protein coding | Homo sapiens RAB9A, member RAS oncogene family (RAB9A), transcript variant 1, mRNA. (from RefSeq NM_004251) |
| **TRAPPC2** | 137122243 | 137344635 | - | ENSG00000196459.13 | Protein coding | Homo sapiens trafficking protein particle complex 2 (TRAPPC2), transcript variant 2, mRNA. (from RefSeq NM_014563) |
| **OFD1** | 137344744 | 137669353 | + | ENSG00000046651.14 | Protein coding | Homo sapiens oral-facial-digital syndrome 1 (OFD1), mRNA. (from RefSeq NM_003611) |
| **GPM6B** | 137701455 | 139338638 | - | ENSG00000046653.14 | Protein coding | Homo sapiens glycoprotein M6B (GPM6B), transcript variant 4, mRNA. (from RefSeq NM_001001994) |
| **RP1-122K4.2** | 139505392 | 139603904 | + | ENSG00000233535.1 | lincRNA | RP1-122K4.2 (from geneSymbol) |
| **RP1-122K4.3** | 139800065 | 139808820 | - | ENSG00000212663.2 | lincRNA | RP1-122K4.3 (from geneSymbol) |
| **GPX1P1** | 133778735 | 133779340 | - | ENSG00000197582 | Processed pseudogene | glutathione peroxidase pseudogene 1 [Source:HGNC Symbol;Acc:HGNC:4560] |
| **GS1-526D21.5** | 137815519 | 137815984 | + | | antisense | |
| **PSMA6P2** | 128265840 | 128266833 | + | ENSG00000229083.1 | Processed pseudogene | proteasome subunit alpha 6 pseudogene 2 [Source:HGNC Symbol;Acc:HGNC:39607] |

*The official gene symbol, start and end location of the gene on the X chromosome, direction of transcription (strand), Ensembl gene ID, gene type, and a short description obtained from UCSC table browser are shown. Misc. = miscellaneous.*

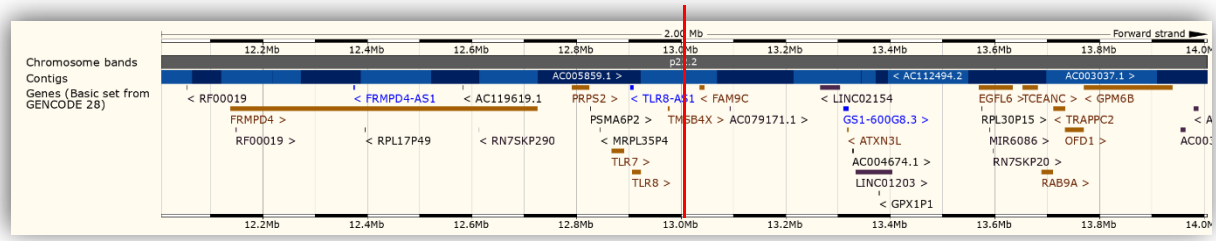


*Figure 5:2 rs850637 genomic region.*
*The genomic region within 1mb of SNP rs850637as obtained from UCSC genome browser. The red vertical line shows the location of rs850637, between TMSB4X and FAM9C at chrX:13005622 (GRCh38.p12).*

## ***Association between rs850637 and gene expression in PBMC***

First, we tested the association between rs850637 (MAF = 44%) and the expression of nearby genes in PBMC of 74 participants, cultured with or without RV16. Out of the 25 genes identified in the vicinity of the variant (Table 5:1, Figure 5:2), 18 genes were expressed in the PBMC samples and so were tested for association with rs850637 (Table 5:2).

*Table 5:2 Association between rs850637 and nearby gene expression*

| Gene | Unstimulated PBMC | | | RV-stimulated PBMC | | |
|---|---|---|---|---|---|---|
| | Effect | *p* | n | Effect | *p* | n |
| ***AC079171.1*** | -0.13 | 0.59 | 35 | -0.16 | 0.54 | 28 |
| ***EGFL6*** | 0.57 | 0.11 | 9 | 0.05 | 0.83 | 36 |
| ***FAM9C*** | -0.22 | 0.38 | 32 | 0.12 | 0.57 | 38 |
| ***GPM6B*** | 0.03 | 0.82 | 73 | -0.25 | 0.08 | 74 |
| ***GPX1P1*** | -0.18 | 0.21 | 73 | -0.08 | 0.59 | 74 |
| ***GS1-526D21.5*** | -0.05 | 0.72 | 68 | -0.12 | 0.43 | 71 |
| ***GS1-600G8.5*** | -0.60 | 0.02 | 26 | -0.22 | 0.14 | 72 |
| ***OFD1*** | 0.29 | 0.04 | 73 | 0.02 | 0.92 | 74 |
| ***PRPS2*** | 0.03 | 0.82 | 73 | 0.17 | 0.25 | 74 |
| ***PSMA6P2*** | -0.07 | 0.71 | 53 | -0.06 | 0.72 | 65 |
| ***RAB9A*** | -0.11 | 0.47 | 73 | -0.001 | 1.00 | 74 |
| ***RP1-122K4.3*** | -0.29 | 0.57 | 8 | -0.03 | 0.96 | 7 |
| ***TCEANC*** | 0.37 | 0.01 | 73 | 0.31 | 0.03 | 74 |
| ***TLR7*** | -0.001 | 0.99 | 73 | 0.19 | 0.20 | 74 |
| ***TLR8*** | -0.19 | 0.19 | 73 | -0.08 | 0.59 | 74 |
| ***TLR8-AS1*** | 0.13 | 0.68 | 14 | -0.01 | 0.97 | 29 |
| ***TMSB4X*** | -0.40 | 0.01 | 73 | -0.30 | 0.04 | 74 |
| ***TRAPPC2*** | 0.08 | 0.61 | 73 | 0.02 | 0.89 | 74 |

*The genes within 1 Mb are tested for association. Shown are the A allele association with the change in mRNA count, p-value of the association (p), and the number of tests performed (n). Unstimulated and RV16-stimulated PBMC samples were tested for an association. Genes underlined with a dash had low expression levels (CPM < 2). None were significant after multiple testing correction (p < 0.05 / 36 tests = 0.001).*

The results in Table 5:2 show that in unstimulated PBMC, the rs850637:A allele was associated with decreased expression of *GS1-600G8.5* and *TMSB4X*, and increased expression of *OFD1* and *TCEANC*, at a nominal significance level of $p < 0.05$. The association with *TCEANC* and *TMSB4X* was also nominally significant after RV16 stimulation. However, *GS1-600G8.5* was lowly expressed (Figure 5:3), and none of these associations remained significant after correcting for the number of genes tested ($p < 0.05 / 36$ tests = 0.001) and so are likely to represent false-positive findings.

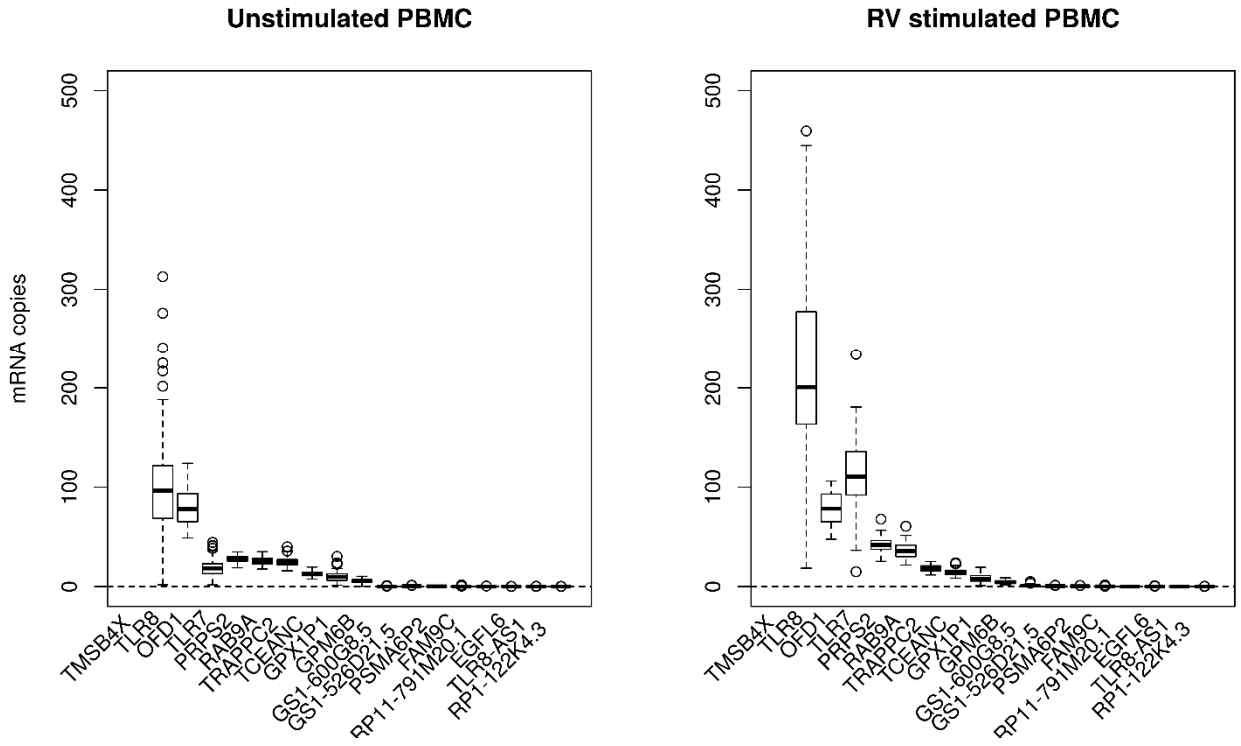


*Figure 5:3 Gene expression of genes near rs850637.*
*The median and interquartile range of mRNA copy number of genes near rs850637 in PBMC. Genes are listed in descending order of median copy number for unstimulated (left) and RV-stimulated PBMC samples (right). Note: TMSB4X mRNA copy number is outside the range visualised.*

### ***Association between rs850637 and gene expression measured in the Genotype-Tissue Expression (GTEx) project***

Next, to expand sample size and investigate the possibility that rs850637 might influence the expression of nearby genes in other relevant tissues, we examined the association between rs850637 and nearby genes using data from the GTEx project. We considered three tissue types relevant for asthma: whole blood (n = 369), lung (n = 383) and spleen (n = 146).

*Table 5:3 Association between rs850637 and nearby gene expression in GTEx dataset*

| | Lung | | Whole blood | | Spleen | |
|---|---|---|---|---|---|---|
| **Gene** | NES | *p*-value | NES | *p*-value | NES | *p*-value |
| ***EGFL6*** | 0.0024 | 0.93 | | | -0.95 | 0.34 |
| ***FAM9C*** | | | | | 0.77 | 0.44 |
| ***FRMPD4*** | -0.015 | 0.77 | | | | |
| ***GPM6B*** | -0.045 | 0.057 | -0.029 | 0.38 | 0.18 | 0.85 |
| ***GPX1P1*** | -0.039 | 0.49 | 0.037 | 0.51 | 0.31 | 0.76 |
| ***GS1-600G8.5*** | 0.027 | 0.58 | | | | |
| ***OFD1*** | -0.02 | 0.48 | -0.023 | 0.38 | -0.43 | 0.66 |
| ***PRPS2*** | 0.024 | 0.43 | 0.03 | 0.21 | -0.75 | 0.45 |
| ***RAB9A*** | 0.0026 | 0.93 | 0.027 | 0.31 | -1.3 | 0.18 |
| ***RP1-122K4.2*** | | | | | -0.33 | 0.74 |
| ***RP1-122K4.3*** | -0.11 | 0.0077 | | | 0.36 | 0.72 |
| ***RP11-142G7.2*** | | | | | 2.2 | 0.027 |
| ***RP11-1L9.1*** | | | | | 1.5 | 0.13 |
| ***RP11-791M20.1*** | | | | | 0.44 | 0.66 |
| ***TCEANC*** | -0.012 | 0.59 | -0.025 | 0.44 | 0.7 | 0.49 |
| ***TLR7*** | 0.029 | 0.3 | 0.021 | 0.35 | -0.37 | 0.71 |
| ***TLR8*** | 0.012 | 0.61 | 0.013 | 0.33 | 0.28 | 0.78 |
| ***TLR8-AS1*** | -0.19 | 0.00024 | -0.025 | 0.43 | -1.4 | 0.16 |
| ***TMSB4X*** | -0.046 | 0.03 | 0.0036 | 0.87 | -1.7 | 0.09 |
| ***TRAPPC2*** | -0.033 | 0.31 | 0.027 | 0.36 | 0.15 | 0.88 |

*Genes within 1mb of SNP rs850637 were tested for an association in the Genotype-Tissue Expression (GTEx) project dataset. The Normalised Effect Size (NES) on gene expression is reported for the A allele; the corresponding p-value is also shown. Whole blood (n = 369), lung (n = 383) and spleen (n = 146) samples were tested. Significant p-value < 0.05 / 44 tests = 0.001 are bolded.*

The antisense gene *TLR8-AS1* showed the most significant association with rs850637 ($p$ = 0.00024; Table 5:3), with the A allele associated with decreased *TLR8-AS1* expression in the lung (Figure 5:4). This association remained significant after accounting for multiple testing ($p$ < 0.05 / 44 tests = 0.001); there were no other associations significant at that threshold.

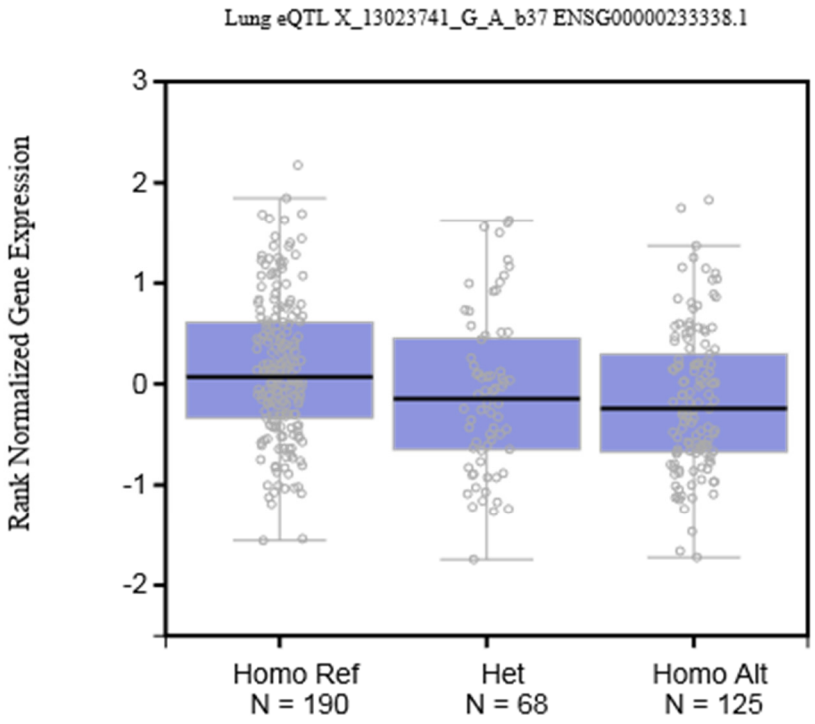


*Figure 5:4 TLR8-AS1 gene expression by rs850637 genotype.*
*Rs850637 genotype association with TLR8-AS1 normalised gene expression levels in GTEx dataset. Homo ref = homozygous or hemizygous for GG/G allele reference genotype, Het = heterozygous for GA allele, and Homo Alt = homozygous or hemizygous for AA/A allele alternative genotype. Graph obtained from GTEx portal.*

### ***Association between rs850637 and gene expression measured in whole blood in the large Framingham Heart Study (FHS)***

The largest eQTL study published to date, that included the X-chromosome, was performed with whole blood samples collected by the Framingham Heart Study (n = 5622). In this dataset, rs850637 was associated with the expression of *TLR7*, with the A allele associated with decreased expression (Table 5:4).

*Table 5:4 Association between rs850637 and TLR7 gene expression in FHS database*

| | Association | | | | |
|---|---|---|---|---|---|
| **Gene** | $R^2$ | Effect | T | *p* | FDR |
| ***TLR7*** | 0.004 | -0.016 | -4.56 | $5.18e^{-6}$ | **1.6e-4** |

*The association $R^2$ score, A allele effect size on TLR7 gene expression, T-value, p-value and false discovery rate (FDR) are shown as reported in the FHS database whole blood samples (n = 5622). Significant FDR < 0.05 is bolded.*

### ***Association between rs850637 and TLR7 and TLR8-AS1 gene expression across three databases***

Table 5:5 compares the association between rs850637 and *TLR7 and TLR8-AS1* gene expression across the three datasets. *TLR8-AS1*, that was significantly associated with rs850637 in the GTEx database, had low expression in our own dataset (Figure 5:3) and was not reported in the FHS dataset. The association between rs850637 and *TLR7* gene expression was significant only in the FHS dataset.

*Table 5:5 Comparison of significant genes across the three studies for A allele*

| ***TLR8-AS1*** | | | | |
|---|---|---|---|---|
| | Study | Tissue | Effect | *p* |
| | **Our samples** | PBMC: Unstim. | 0.13 | 0.68 |
| | **Our samples** | PBMC: RV | -0.01 | 0.97 |
| | **GTEx** | Lung | -0.19 | **0.00024** |
| | **GTEx** | Whole blood | -0.025 | 0.43 |
| | **GTEx** | Spleen | -1.4 | 0.16 |

| ***TLR7*** | | | | |
|---|---|---|---|---|
| | Study | Tissue | Effect | *p* |
| | **Our samples** | PBMC: Unstim. | -0.001 | 0.99 |
| | **Our samples** | PBMC: RV | 0.19 | 0.20 |
| | **Our samples** | Whole blood | 0.11 | 0.18 |
| | **GTEx** | Lung | 0.029 | 0.30 |
| | **GTEx** | Whole blood | 0.021 | 0.35 |
| | **GTEx** | Spleen | -0.37 | 0.71 |
| | **FHS** | Whole blood | -0.016 | **5.18E-06** |

*The study, sample tissue type used, the effect size for rs850637 A allele on TLR8-AS1 or TLR7 gene expression and association p-value are shown. Significant p-values are bolded.*

### 5.3.3. Association between rs850637 and TLR7/TLR8 receptor function

To assess if the SNP rs850637 affects TLR7/8 function, IFNα was used as a marker of TLR7 function, and TNF and IL12 as a marker of TLR8 function. First, we tested the association between rs850637 and protein levels of these three cytokines, measured in PBMC samples after TLR7/8 activation with RV16 or agonists stimulation from 238 individuals. We found no significant associations with rs850637 (Table 5:6).

*Table 5:6 rs850637 associations with cytokines produced in PBMC.*

| Cytokine | Stimulus | Effect | *p*-value | n |
|---|---|---|---|---|
| **IFNα** | RV16 | -0.049 | 0.55 | 238 |
| **IFNα** | TLR7 agonist | 0.039 | 0.64 | 238 |
| **TNF** | TLR8 agonist | -0.046 | 0.58 | 238 |
| **IL12** | TLR8 agonist | 0.011 | 0.89 | 238 |

*PBMC were stimulated to activate TLR7/8 and IFNα, TNF and IL12 measured. Results for rs850637 allele A association with normalised cytokine production levels and the corresponding p-value are shown.*

Next, we tested associations between the SNP and the expression of the genes that encode these cytokines, in unstimulated and RV16-stimulated PBMC samples from 74 individuals. We found a nominally significant association with the expression of *IFNA* in RV-stimulated samples, but this did not survive correction for multiple testing ($p < 0.05 / 3$ tests $= 0.016$).

*Table 5:7 Associations between rs850637 and cytokine gene expression.*

| | Unstimulated PBMC | | | RV16-stimulated PBMC | | |
|---|---|---|---|---|---|---|
| **Gene** | **Effect** | ***p*-value** | **n** | **Effect** | ***p*-value** | **n** |
| ***IFNA* genes** | 0.042 | 0.78 | 72 | 0.24 | 0.021 | 74 |
| ***TNF*** | -0.003 | 0.99 | 72 | 0.11 | 0.45 | 74 |
| ***IL12A*** | 0.12 | 0.40 | 72 | 0.048 | 0.75 | 74 |

*The PBMC were left unstimulated or stimulated with RV16. Results for rs850637 allele A association mRNA count and the corresponding p-value are shown. None were statistically significant after multiple testing correction ($p < 0.05 / 3$ tests $= 0.016$).*

### 5.3.4. Association between rs850637 and clinical variables

Next, we tested for an association between rs850637 and (1) self-reported cold frequency; and (2) immune cell counts. No association was seen between rs850637 and cold frequency. After correcting for multiple testing ($p < 0.05 / 7$ tests $= 0.007$), rs850637 was associated with basophil numbers in whole blood (Table 5:8), with the A allele associated with higher counts. A more modest association was observed between rs850637 and eosinophil numbers in whole blood; however, this was not significant after multiple testing correction.

*Table 5:8 rs850637 associations with cold frequency and immune cell counts.*

| Outcome | Effect | *p*-value | n |
|---|---|---|---|
| **Cold Frequency** | -0.10 | 0.22 | 226 |
| **Total White Blood Cells** | 0.020 | 0.80 | 236 |
| **Platelets** | 0.062 | 0.45 | 235 |
| **Neutrophils** | -0.070 | 0.39 | 236 |
| **Lymphocytes** | 0.14 | 0.073 | 236 |
| **Monocytes** | 0.15 | 0.073 | 236 |
| **Eosinophils** | 0.17 | 0.017 | 236 |
| **Basophils** | 0.22 | 0.0044 | 236 |

*Results for rs850637 allele A association with the self-reported cold frequency and immune cell counts in whole blood and the corresponding p-value are shown. Significant p-value < 0.05 / 7 tests = 0.007 are bolded.*

### 5.3.5. Association between other SNPs in/near *TLR7/TLR8* and gene expression

Next, we expanded the association analysis to other SNPs within 1 Mb of *TLR7/8* genes. We first tested if SNPs in/near *TLR7/TLR8* have an association with *TLR7* and/or *TLR8* expression. Gene expression levels were measured in whole blood (n = 238) using RT-PCR, and in PBMC (n = 74) with RNA-seq. PBMC were cultured with or without RV16.

We tested the association between *TLR7/8* expression and a total of 1748 SNPs. The strongest SNP associations observed for each gene and biological sample type are shown in Table 5:9. None of these associations exceeded the *p*-value threshold required to correct for multiple SNP testing, specifically $p$ = 5.2e-05 for whole blood and $p$ = 3.6e-05 for PBMC samples. Using these relatively stringent thresholds, there were no significant associations between SNPs in/near *TLR7/8* and the expression of either of these genes, though the study may not have had significant statistical power.

*Table 5:9 Top association between TLR7/8 SNPs and gene expression in whole blood and PBMC*

| Gene Expression | | | Top Genomic Marker | | | | Association | |
|---|---|---|---|---|---|---|---|---|
| **Gene** | Sample | n | SNP ID | Effect Allele | Allele Freq. | Position | Effect | *p* |
| **TLR7** | WB unstim. | 236 | rs178997 | T | 0.19 | 12900792 | 0.39 | 0.0004 |
| **TLR7** | PBMC unstim. | 73 | rs12382299 | G | 0.42 | 12929275 | 0.50 | 0.0020 |
| **TLR7** | PBMC RV | 74 | rs6640964 | C | 0.28 | 12300973 | -0.58 | 0.0033 |
| **TLR8** | WB unstim. | 236 | rs5935410 | T | 0.16 | 12843546 | -0.38 | 0.0002 |
| **TLR8** | PBMC unstim. | 73 | rs9780695 | A | 0.14 | 12981663 | 0.63 | 0.0003 |
| **TLR8** | PBMC RV | 74 | rs6640986 | C | 0.42 | 12422688 | -0.44 | 0.0027 |

*Gene expression was measured at baseline in unstimulated whole blood and PBMC samples and RV16-stimulated PBMC samples. The top genomic marker and its effect allele, effect allele frequency, and position are shown. The allele association with gene expression and p-value are described. None were significant after multiple testing correction p < 5.2e-05 for whole blood and p < 3.6e-05 for PBMC.*

### 5.3.6. Association between SNPs in/near *TLR7/TLR8* and receptor function

Next, to assess whether genetic variation in the *TLR7/8* gene region could affect the function of those receptors, we used cytokine production in response to receptor activation as a proxy. We measured IFNα, which is produced in response to TLR7 activation and TNF and IL12 that are produced in response to TLR8 activation. We tested the association between SNPs and both protein and RNA levels for these three cytokines, measured in PBMC stimulated for 24 h with TLR7/8 receptor activators.

Table 5:10 depicts the strongest SNP associations with cytokine protein levels. No individual association exceeded the *p*-value threshold required to correct for multiple testing, which was $p$ = 2.3e-5 (correcting for the number of SNPs and cytokines). As such, within the constraints of study power, there was insufficient evidence for significant associations between the SNPs tested and protein levels of these three cytokines.

*Table 5:10 Top associations between TLR7/8 SNPs and cytokine production.*

| Cytokine Production | | | Top Genomic Marker | | | | Association | |
|---|---|---|---|---|---|---|---|---|
| **Cytokine** | Stimulus | n | SNP ID | Effect Allele | Allele Freq. | Position | Effect | *p* |
| **IFNα** | RV16 | 238 | rs1006239 | C | 0.72 | 12779171 | 0.33 | 0.0011 |
| **IFNα** | TLR7 agonist | 238 | rs5935415 | C | 0.13 | 12846581 | 0.36 | 0.0037 |
| **TNF** | TLR8 agonist | 238 | rs4830472 | T | 0.75 | 12695401 | 0.28 | 0.0003 |
| **IL12** | TLR8 agonist | 238 | rs5935405 | C | 0.26 | 12836807 | 0.36 | 0.0004 |

*IFNα, TNF and IL12 production were measured in TLR7/8 stimulated PBMC samples. The top genomic marker and its effect allele, effect allele frequency, and position are shown. The allele association with cytokine production and p-value are described. None were significant after multiple testing correction p < 2.3e-5.*

Results from the association analysis between SNPs and gene expression levels for the same three cytokines are shown in Table 5:11. Gene expression was measured in unstimulated and RV-stimulated PBMC (n = 74). There are 17 different gene products for IFNα, 11 of which were quantified in the RNA-seq data and summed for the analysis. Similarly, IL12 consists of two subunits transcribed by two genes: *IL12A* and *IL12B*. *IL12B* was not quantified in our data; therefore, only *IL12A* was analysed. There were no significant associations between SNPs and gene expression levels for these cytokines after accounting for multiple testing (*p*-value threshold of 1.9e-5; Table 5:11).

*Table 5:11 Top associations between TLR7/8 SNPs and cytokine gene expression.*

| Cytokine Gene Expression | | | | | | Top Genomic Marker | | | | Association | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Gene** | PBMC Sample | n | Chr | Start | End | SNP ID | Effect Allele | Allele Freq. | Position | Effect | *p* |
| ***IFNA* genes** | Unstim. | *25* | 9 | | | rs1266352 | T | 0.19 | 12767037 | 1.02 | 0.0028 |
| ***IFNA* genes** | RV16 | *70* | 9 | | | rs2532610 | A | 0.25 | 12781526 | 0.61 | 0.0004 |
| ***TNF*** | Unstim. | *73* | 6 | 31575566 | 31578336 | rs56236583 | G | 0.10 | 12592368 | -0.47 | 0.0011 |
| ***TNF*** | RV16 | *74* | 6 | 31575566 | 31578336 | rs141566161 | T | 0.04 | 12683149 | 1.00 | 0.0011 |
| ***IL12*** | Unstim. | *73* | 3 | 159988749 | 15999 6012 | rs6640970 | A | 0.23 | 12311026 | -0.51 | 0.0059 |
| ***IL12*** | RV16 | *74* | 3 | 159988749 | 15999 6012 | rs5934027 | T | 0.24 | 12677807 | -0.39 | 0.0048 |

*Gene expression was measured at baseline in unstimulated PBMC and in RV16-stimulated PBMC. The top genomic marker and its effect allele, effect allele frequency, and position are shown. The allele association with gene expression and p-value are described. None were significant after multiple testing correction p < 1.9e-5.*

### 5.3.7. Association between SNPs in/near *TLR7/TLR8* and clinical variables

Since many respiratory viruses are ssRNA viruses that are recognised by TLR7 and TLR8, we hypothesised that nearby SNPs might be associated with the frequency of colds. All participants reported how often they suffered from upper respiratory infections (cold frequency). Immune cell counts are thought to influence antiviral responses, and so were also tested for an association with SNPs in/near *TLR7/TLR8*. The strongest association between SNPs and cold frequency is shown in Table 5:12, which was not significant after accounting for multiple testing (*p*-value threshold of 0.0001; Table 5:12).

*Table 5:12 Top association between TLR7/8 SNPs and self-reported cold frequency.*

| | | Top genomic marker | | | | Association | |
|---|---|---|---|---|---|---|---|
| | n | SNP ID | Effect Allele | Allele Freq. | Position | Effect | *p* |
| **Cold frequency** | 226 | rs9781757 | C | 0.66 | 12988623 | 0.25 | 0.0017 |

*The top genomic marker and its effect allele, allele frequency, and position are shown. The allele association with the self-reported cold frequency and the corresponding p-value are shown. None were significant after multiple testing correction p < 0.0001.*

The results from the association analysis between SNPs and immune cell counts are reported in Table 5:13. Immune cell counts were measured from whole blood (n = 236) using pathology services. There were no significant associations between SNPs and immune cell count after accounting for multiple testing (*p*-value threshold of 1.3e-05 correcting for the number of SNPs and cell counts; Table 5:13).

*Table 5:13 Top associations between TLR7/8 SNPs and immune cell counts.*

| Cell Count | | Top Genomic Marker | | | | Association | |
|---|---|---|---|---|---|---|---|
| | n | SNP ID | Effect Allele | Allele Freq. | Position | Effect | *p* |
| **Total White Blood Cells** | 236 | rs2699997 | T | 0.15 | 12807077 | -0.27 | 0.0045 |
| **Platelets** | 235 | rs6639207 | G | 0.60 | 12607691 | -0.28 | 0.0006 |
| **Neutrophils** | 236 | rs5935411 | C | 0.13 | 12843694 | -0.39 | 0.0021 |
| **Lymphocytes** | 236 | rs1483191 | T | 0.28 | 12979350 | 0.23 | 0.0033 |
| **Monocytes** | 236 | rs5979778 | G | 0.24 | 12965184 | 0.23 | 0.0033 |
| **Eosinophils** | 236 | rs2074110 | T | 0.22 | 13015959 | 0.32 | 0.0001 |
| **Basophils** | 236 | rs955279 | A | 0.34 | 12976131 | 0.26 | 0.0009 |

*The top genomic marker and its effect allele, allele frequency, and position are shown. The allele association with immune cell counts and the corresponding p-value are shown. None were significant after multiple testing correction p < 1.3e-05.*

### 5.3.8. Top SNP associations in larger datasets

We identified the top SNPs that associated with *TLR7/8* gene expression, cytokine production or immune variables and searched in the larger databases for an association between those SNPs and *TLR7*, *TLR8*, and *TLR8-AS1* expression. Out of 24 top SNPs, seven were significant eQTLs for these genes in the larger GTEx (lung, n = 383) and Framingham heart study (whole blood, n = 5622) databases. Seven SNPs had been identified from associations with *TLR7/8* baseline expression, immune cell count and cold frequency (Table 5:14). Asthma risk SNP rs850637 was in high linkage disequilibrium D' > 0.8 with the three closest SNPs (rs1483191, rs9780695, rs9781757) and in moderate linkage disequilibrium D' > 0.4 with the other three SNPs (rs12382299, rs5979778, rs955279) (Figure 5:5).

*Table 5:14 eQTLs between top SNPs from association tests and TLR7/TLR8/TLR8-AS1 genes.*

| Top Genomic marker | | | Gene | Association | | | |
|---|---|---|---|---|---|---|---|
| **Association** | SNP ID | Position | Symbol | Effect | *p* | Tissue | Study |
| **Basophils** | rs955279 | 12994250 | TLR7 | 0.03 | 8.80E-09 | WB | F |
| | rs955279 | 12994250 | TLR8 | 0.01 | 5.85E-05 | WB | F |
| | rs955279 | 12994250 | TLR8-AS1 | 0.26 | 9.40E-07 | L | G |
| **Cold frequency** | rs9781757 | 13006742 | TLR7 | 0.02 | 4.13E-07 | WB | F |
| **Lymphocytes** | rs1483191 | 12997469 | TLR7 | 0.03 | 9.32E-09 | WB | F |
| | rs1483191 | 12997469 | TLR8 | 0.01 | 6.67E-05 | WB | F |
| | rs1483191 | 12997469 | TLR8-AS1 | 0.23 | 1.30E-05 | L | G |
| **Monocytes** | rs5979778 | 12983303 | TLR7 | 0.03 | 3.81E-12 | WB | F |
| | rs5979778 | 12983303 | TLR8 | 0.02 | 3.06E-10 | WB | F |
| | rs5979778 | 12983303 | TLR8-AS1 | -0.33 | 1.80E-10 | L | G |
| **TLR7 PBMC unstim.** | rs12382299 | 12947394 | TLR7 | -0.02 | 1.91E-08 | WB | F |
| **TLR7 whole blood unstim.** | rs178997 | 12918911 | TLR7 | -0.03 | 6.73E-14 | WB | F |
| | rs178997 | 12918911 | TLR8 | -0.01 | 2.42E-08 | WB | F |
| | rs178997 | 12918911 | TLR8-AS1 | 0.36 | 1.10E-07 | L | G |
| **TLR8 PBMC unstim.** | rs9780695 | 12999782 | TLR8 | -0.04 | 1.06E-34 | WB | F |
| | rs9780695 | 12999782 | TLR7 | -0.05 | 1.96E-18 | WB | F |

*The association type with the top genomic marker, the genomic marker and its position, significant target gene, allele association with target gene expression, corresponding p-value, the study database, and tissue sample type are shown. G = GTEx dataset F = FHS study, L = lung, WB = whole blood.*

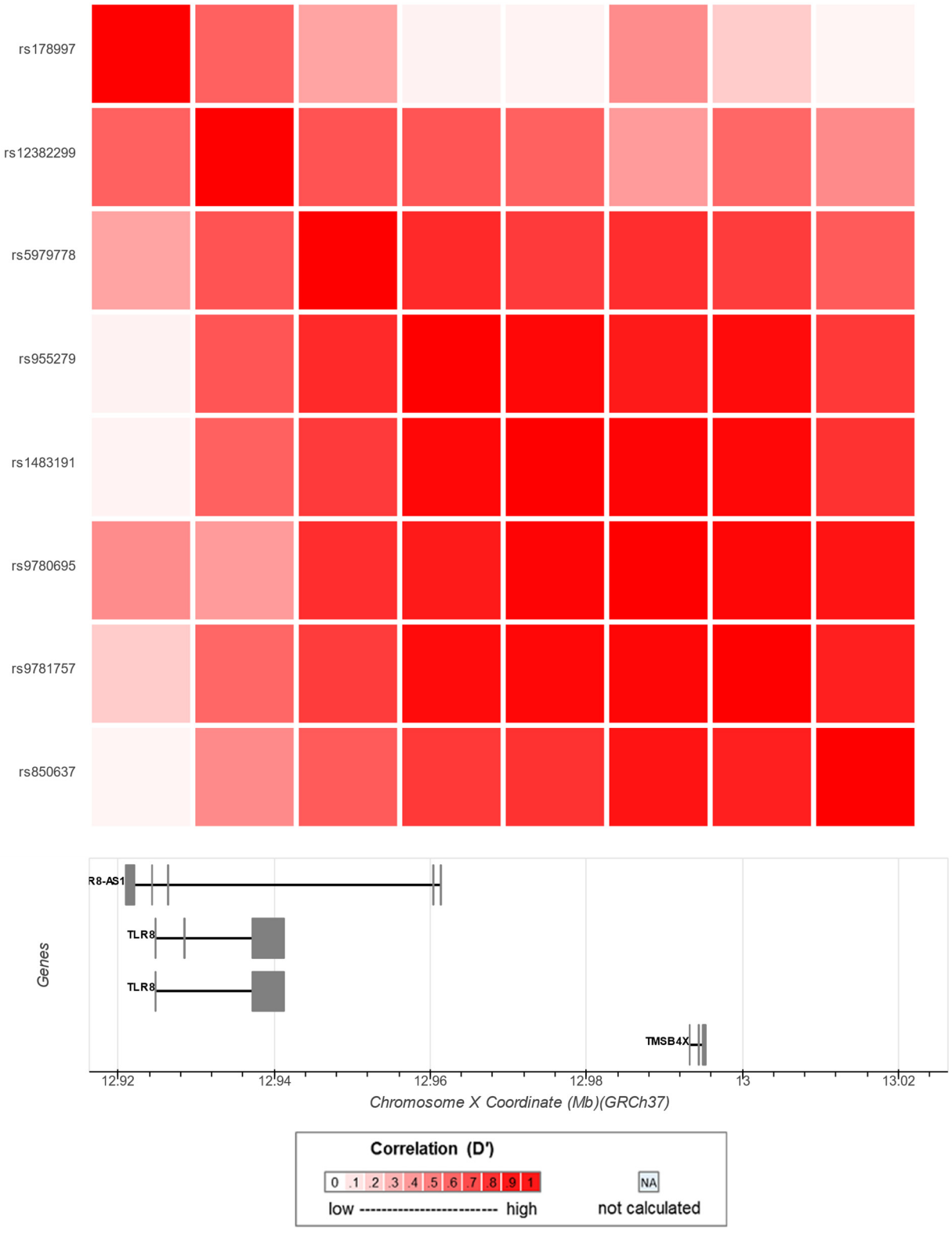


*Figure 5:5 Linkage disequilibrium D' score matrix between the top SNPs and rs850637. Obtained from LDlink.*

## 5.4. Discussion

Important findings from Chapter 3 were that *TLR7* gene expression on circulating leukocytes was lower in asthma and was associated with frequent respiratory infections in men. Those results align with the previous reports that TLR7 has an important role in various immunological conditions, including asthma (Pritchard et al. 2014; Roponen et al. 2010; Rupani et al. 2016). TLR7 and TLR8 are in the centre of the crossover between antiviral immunity and type 2 immunity related to asthma, and assessing their role is critical for understanding antiviral immunity in asthma. Therefore, here we evaluate the impact of variation in the *TLR7* and *TLR8* gene region on their function and other related clinical variables. Specifically, this study examined the association between polymorphisms in the *TLR7/8* gene region and TLR7/8 gene expression and function, cold frequency, and immune cell counts. The key findings to emerge from this chapter were that SNP rs850637, located in the TLR7/8 locus and recently reported to be a risk factor for asthma, was associated with the expression of *TLR7* and *TLR8-AS1*, and with blood basophil cell count.

Our study included 238 people of confirmed European ancestry, with transcriptome data obtained from a subset of 74 unstimulated and RV16-stimulated PBMC samples. The investigation focused on the function of the SNP rs850637 in the available samples and further expanded to search associations in two publicly available databases: GTEx and Framingham Heart Study (FHS).

Polymorphisms have the potential to affect nearby gene transcription by altering gene promoter regions or the binding of transcription factors to enhancer and silencer sequences. We did not detect associations between rs850637 and the expression of nearby genes in our samples, but in further investigations from the publicly available GTEx eQTL database, we found a significant negative association between rs850637:A and an antisense RNA gene *TLR8-AS1* in the lung. *TLR8-AS1* is a short non-coding gene that shares the sequence with *TLR8* but is transcribed in the opposite direction. The specific function of *TLR8-AS1* is unknown, but it has been identified in a study searching for lncRNAs predicting breast cancer survival (Fan, Ma & Liu 2018), suggesting that it has a functional role. The current consensus is that antisense genes have an important function regulating the gene expression of their corresponding sense genes (Pelechano & Steinmetz 2013), and in this context, it is possible that *TLR8-AS1* may inhibit *TLR8* expression, though this has not yet been proven.

In the FHS, the largest gene expression database that we had access to, we observed a negative association between the rs850637:A asthma-protective allele and *TLR7* gene expression in whole blood. Although, the effect size of the association between rs850637 and *TLR7* expression was small, together with the negative association with *TLR8-AS1* gene expression, these results suggest that rs850637 affects the transcription rates of nearby genes. If reduced *TLR7* gene expression results in reduced TLR7 receptor expression in the cell, then the rs850637:A allele could potentially influence the IFNα-inducing function of TLR7.

Contrary to what was expected from the rs850637 association with *TLR7* gene expression, the results outlined in this chapter indicated a positive association between rs850637:A and *IFNA* gene expression in RV16-activated PBMC (Table 5:7). However, this association was not significant after correcting for multiple testing and was not confirmed using IFNα cytokine production as the outcome. There was no association between rs850637 and *IFNA* gene expression levels at baseline, which are negligible without immune stimulus (Gough et al. 2012). We lacked data on TLR7 agonist-induced *IFNA* gene expression; however, the literature indicates that the great majority of RV-induced IFNα is mediated by TLR7 activation, justifying the use of RV16-induced *IFNA* gene expression as a measurement of TLR7 function (Hatchwell et al. 2015; Xi et al. 2015). Even though our results indicate that rs850637 does not affect the TLR7 function as assessed by IFNα production, this should be validated in larger sample sizes, since in our study population we were not able to reproduce the association observed in the FHS between the SNP and *TLR7* gene expression. One possibility is that other TLR7 functions mediate the association between rs850637 and asthma and this is unrelated to TLR7-induced IFNα production. TLR7 is known to also induce proinflammatory cytokine and antimicrobial peptide production (Saitoh, S-I et al. 2017; Triantafilou et al. 2011; Uehara et al. 2007) and crossregulate IgE responses with the IgE receptor FcεRI on pDC (Gill et al. 2010; Moisan et al. 2006). Future studies should address how rs850637 affects TLR7 function in asthma.

We were unable to test for a correlation between *TLR7* and *TLR8-AS1*, due to the low mRNA copy numbers in our samples, but it is possible that these two genes are associated. Considering that the rs850637 association with *TLR8-AS1* was observed only in lung tissue, it may also be very relevant to asthma. In GTEx, the highest expression of *TLR8-AS1* is observed in lung, followed by spleen and whole blood. *TLR8* gene expression is limited to

those tissues, supporting the theoretical regulatory relationship between these two genes. A possible mechanism of how *TLR8-AS1* could indirectly regulate TLR7 activity is through TLR8-mediated downregulation of TLR7, which has been described in mice (Demaria et al. 2010; Tran, Manzin-Lorenzi & Santiago-Raber 2015) and in human cells (Wang, J et al. 2006). TLR7 and TLR8 are co-expressed only in the lung epithelial cells, monocytes and neutrophils restricting the TLR8-mediated regulation to only those cells. Future research needs to define how the rs850637 SNP affects *TLR7* and *TLR8-AS1* transcription and how *TLR8-AS1*, in turn, affects *TLR7* and *TLR8* gene expression and function.

Interestingly, biological models show that TLR7/8 activation improves asthma phenotype (Drake et al. 2012), which have led to the development of TLR7 agonists as asthma treatment (Junt & Barchet 2015). The results in Chapter 3, showing lower TLR7 gene expression in asthma patients than controls, support the use of those medications, whereas, the observation that the rs850637:G asthma-risk allele was associated with increased TLR7 gene expression suggests that a subgroup of asthma patients with the risk SNP may not benefit from treatment activating TLR7 receptor. However, the difference on *TLR7* gene expression change was small between the alleles and the results in Chapter 3 and previous literature indicate an important environmental impact on *TLR7* gene expression; therefore it is possible that the allergic type 2 cytokine milieu, sex hormones and obesity may obscure any genetic predisposition to high TLR7 gene expression.

We also showed that the rs850637:A allele significantly associated with increased basophil counts in our study samples. One possible explanation for this observation is that the SNP modifies the cytokine or chemokine environment induced by TLR7 and TLR8 activation and this, in turn, alters the production and trafficking of basophils, and perhaps other immune cell populations. Basophils are a type of granulocyte that produces histamine, serotonin and heparin and can induce inflammation. The role of basophils in asthma has not received as much attention as eosinophils but is well documented (Fux & Garnier 2017). Basophils are known to be an effector cell that interacts with innate lymphoid cell (ILC)2 in establishing type 2 inflammation (Motomura et al. 2014). Importantly, basophils appear to induce type 2 inflammation in response to RV-infection, indicating a possible role for basophils in balancing the antiviral and type 2 immunity in asthma (Agrawal, R et al. 2014; Leffler et al. 2018). When establishing the connection between the SNP and asthma, the functional

relevance of basophils in establishing type 2 inflammation in asthma and whether it is TLR7-mediated should be examined further.

Among the most significant SNPs in the *TLR7/8* region tested for associations, none were repeatedly the most significant across the tests performed but several were in high linkage disequilibrium with each other and may act as proxy for each other. The SNPs rs955279 and rs1483191 were both identified for most significant association with blood leukocytes, rs955279 for blood leukocyte count and rs1483191 for blood basophil count. The SNPs rs2532610 and rs1006239 were both most associated with different IFNα measures. The SNPs rs5935410 and rs5935405 were identified in most significant association with *TLR8* gene expression and TLR8-induced IL-12 production, respectively. Despite not surviving the multiple testing adjustment, these SNP pairs in linkage disequilibrium and their similar associations could present candidate SNPs for further study. Additionally, the SNP rs178997 that was most associated with increased *TLR7* gene expression at baseline and is located in between *TLR7* and *TLR8* might present a prospective candidate for variant affecting *TLR7* gene expression.

An important caveat of this study was its small sample size, which in practice provided adequate power to identify only relatively large SNP effects on gene expression and cytokine levels. To supplement the lack of power, we identified the most significant SNPs for each association test and searched for their eQTLs in the external GTEx and FHS databases. Out of those SNPs, seven were significant modifiers of *TLR7*, *TLR8* or *TLR8-AS1* gene expression and six in linkage disequilibrium with rs850637, suggesting that the rs850637 haplotype may be responsible for the effect on *TLR7/8/8-AS1* gene expression.

An important consideration for the study of TLR7 and TLR8 is their reported escape from XCI (Chung et al. 2006; Souyris et al. 2018). Souyris et al. (2018) findings indicate that approximately 20% of females express both *TLR7* gene copies. However, because the overexpression of *TLR7* and *TLR8* have more commonly been associated with autoimmune disease and their reduced function is more common in asthma, it is less likely that the XCI escape affects asthma patients. Additionally, because 80% of females were shown to silence one gene copy successfully and *TLR7* or *TLR8* gene expression difference was not significant between the sexes in our study at baseline (Figure 5:1), adjusting for XCI escape was regarded as unnecessary, and both sexes were combined in the analysis.

In conclusion, the data presented here links the asthma risk variant rs850637 to the expression of *TLR7* and *TLR8-AS1*, as well as basophil cell count. The possibility that these variables are interlinked should be further investigated by assessing the potential targets of *TLR8-AS1* and by further studying the transcriptomic profile of basophils in asthma.

# Chapter 6: Conclusion and future directions

## 6.1. Discussion

This thesis aimed to investigate self-reported respiratory infections and antiviral immune function and how these are associated with:

- clinical and demographic parameters,
- variations in TLR7/8 expression and function, and whether these are genetically regulated, and
- the presence of asthma and asthma severity.

The main motive for the study were previous reports of antiviral immune deficiencies, in asthma, specifically poor IFNα production, thought to contribute to severe respiratory infections and asthma exacerbation susceptibility during respiratory viral infections. To further study those deficiencies, in Chapter 3, we determined what clinical and immunological parameters are associated with self-reported respiratory infection frequency, the presence of asthma, asthma severity and control. Interestingly, we discovered that the factors associated with self-reported cold frequency vary with gender. In males, but not in females, cold frequency was independently associated with baseline *TLR7* and *CLEC4C* gene expression, whereas in females, cold frequency was independently associated with age and BMI, such that even a modest increase in weight was associated with a greater frequency of self-reported colds.

Asthma patients had lower baseline *TLR7* gene expression than controls, and those with poor asthma symptom control had low *TLR8* gene expression independently of *TLR7* gene expression, sex and BMI. In women, asthma was associated with high BMI and in men with a modest change in the TNF response to *TLR8* activation. Contrary to many previous reports, we did not find that asthma was associated with a systematic deficiency rhinovirus (RV)-stimulated IFNα production.

To elucidate what factors are critical for an efficient antiviral IFNα response, in Chapter 4, we contrasted the baseline transcriptomes of high and low IFNα producer groups. We discovered that many innate immune variables are upregulated in the high IFNα producers. We also observed that low IFNα production was associated with oxidative stress at baseline and antibacterial peptide production during viral infection. Finally, we studied the consequences of genetic variation in the *TLR7/8* gene region. We learnt that the asthma-

associated rs850637 SNP is associated with *TLR7* and *TLR8-AS1* gene expression and basophil blood count but not with TLR7/8 cytokine-inducing function.

Since the evidence emerged over 20 years ago that asthma patients are prone to respiratory virus-induced exacerbations (Johnston et al. 1995; Nicholson, Kent & Ireland 1993), an accumulating body of research has aimed to address the reasons why. Consistent with reports of immune deficiency in asthma, we observed a higher rate of respiratory infections in asthma patients than controls, which was not linked to asthma symptom severity or control. Contrary to expectations, we detected no consistent difference in the RV16-stimulated IFNα response between the asthma cases and controls. A few recent studies have also challenged the notion that the antiviral immune response in asthma is characterised by type I IFN deficiency. Two transcriptomic studies show that type I IFN production varies between different types of respiratory infection-associated asthma exacerbations but is not necessarily low (Gomez et al. 2018; Khoo et al. 2019). The third birth-cohort study investigated the association between various cytokine responses to RV and childhood asthma and found that participants with the highest and lowest IFN production were most likely asthmatic compared to other cytokine response types. The distinction between those two groups was the onset of asthma (Custovic et al. 2018). Our results, together with the earlier reports, suggest that type I IFN deficiency is associated with only a subgroup of asthma patients. The previous reports and recently published data provide support for the hypothesis that two asthma phenotypes exist, one with low type I IFN response and one with high type I IFN response. The low type I IFN response is associated with the most severe asthma exacerbations and type 2-mediated immune responses, whereas a high type I IFN response is associated with less complicated asthma exacerbations and perhaps ILC2-mediated type 2 inflammation based on the high type 2 immune response common to both groups in the Custovic et al. (2018) report.

Instead, Chapter 3 showed that baseline TLR7 gene expression, rather than type I IFN, was significantly lower in the asthma group than the healthy group. This is interesting, given that TLR7 activation is thought to be the main source of IFNα in RV infection, and that we were unable to confirm IFNα deficiency in asthma. One possible explanation is that TLR7 exerts key functions that extend beyond IFNα induction. For example, during viral infection, the main function of TLR7 is to induce IFNα, that function is mostly driven by pDCs that are a minor cell population, yet TLR7 is expressed in other cell types. Other cell types are more

biased towards proinflammatory, and antimicrobial peptide production and even pDC produce proinflammatory cytokines after TLR7 stimulation before pDC clustering takes place (Agrawal, S & Gupta 2011; Saitoh, S-I et al. 2017; Uehara et al. 2007). Further, some evidence exists of the counterregulatory relationship between TLR7 and type 2 inflammation in mouse studies. One study shows that depletion of TLR7 in mice increases a population of myeloid-derived suppressor cells (MCDS) in the lungs, which contributes to Th2 cell and cytokine bias (Jeisy-Scott et al. 2011). Further evidence suggests that TLR7-activation in B-cells and pDC suppresses the asthma-like phenotype and type 2 responses through the activation of Tregs (Khan et al. 2015; Lynch et al. 2018; Pham Van et al. 2011). Treg activation by pDC was further shown to be important for the prevention of asthma and bronchiolitis in a mouse model, suggesting a possible role in asthma pathogenesis (Lynch et al. 2018). In addition to Tregs, TLR7 can also suppress IL-17 secreting Th17 cells (Lukacs et al. 2010; Vultaggio et al. 2011; Ye et al. 2017). Th17 cytokine responses are of particular importance to IL17-high severe asthma type that associated with neutrophilic asthma inflammation providing another link between asthma and TLR7 (Chesné et al. 2014).

In Chapter 5, we investigated if genetic variation could contribute to the reduced TLR7 gene expression. An association between TLR7 and asthma has recently been supported by the novel finding from Ferreira et al. (2019) that the single nucleotide polymorphism (SNP) rs850637 in the *TLR7/8* gene region protects from asthma. The data presented in Chapter 5 showed that the rs850637 SNP was associated with reduced TLR7 gene expression and increased basophil count in people with the asthma protective allele. Those effects were opposite to what we expected as basophils are often reported to contribute to asthma inflammation (Salter et al. 2016). Previous literature has provided evidence that TLR7 might be associated with asthma based on the findings of *TLR7* genetic alterations in asthma patients (Moller-Larsen et al. 2008; Nilsson et al. 2012; Zhang, Q et al. 2015) and small scale studies showing low TLR7 function in the lungs of severe asthma patients (Rupani et al. 2016; Shikhagaie et al. 2014) and in the PBMC of allergic asthma patients (Pritchard et al. 2014; Roponen et al. 2010). In Chapter 3, we show with a large number of study participants that the baseline *TLR7* gene expression reduction in asthma patients is not associated with asthma severity or control. What these contradictory findings may indicate, is that TLR7 expression is mostly under environmental (rather than genetic) influence or that a TLR7-high subpopulation of asthma patients exists, perhaps with similar characteristics to the IFN-high asthma subpopulation.

In contrast, we found baseline *TLR8* gene expression is inversely correlated with asthma control, raising the possibility that TLR8 may have important regulatory properties, including its reported capacity to regulate TLR7 function (Desnues et al. 2014; Wang, J et al. 2006). TLR8 agonists have been trialled for allergy and allergic rhinitis (Horak 2011), but these results indicate a possible role for asthma symptom control, too. TLR8-induced cytokines promote Th1 polarisation, a function which may be antagonised by Th2 inflammation, which is abundant in poorly controlled asthma. Interestingly, the asthma SNP rs850637 that we investigated in Chapter 5 was also associated with decreased *TLR8-AS1* gene expression. Antisense genes are thought to modulate their complementary genes, suggesting that genetic variation may also indirectly alter *TLR8* gene expression via *TLR8-AS1*. Thus far, supporting evidence for TLR8's role in asthma is scarce and hindered by the divergence of mouse and human TLR8 functions. Despite the shared similarity between TLR7/8, they appear to have distinct roles in innate immunity, and our results suggest that further work needs to be done to better understand TLR8 biology in asthma before considering TLR8 agonists for reducing airway inflammation.

Low baseline *TLR7* gene expression was also associated with more frequent respiratory infections in men, as reported in Chapter 3. *TLR8* expression was not associated with respiratory infection frequency; however, its proinflammatory cytokine repertoire activates the adaptive antiviral immune response, whereas TLR7 induces IFN-I production to activate the innate immune response in all cell types to impede viral reproduction. Thus, efficient TLR7 function is expected to have a greater role in reducing the development of symptomatic infection and not TLR8. In contrast to cold prevention, in Chapter 4 we found that the baseline gene expression of both *TLR7* and *TLR8* is important for robust IFNα response, suggesting that both receptors are necessary during the efficient antiviral immune response. The reduced baseline *TLR7* gene expression that contributes to the frequent colds could be attributed to genetic variation such as the SNP rs850637 that reduces *TLR7* gene expression, although we did not see a direct association between this SNP and cold frequency in Chapter 5. The effect size of the SNP rs850637 A allele on TLR7 gene expression was small in the external database with $R^2 = 0.004$ and our study participants had a good representation of all rs850637 alleles. The lack of association between the SNP and cold frequency suggests that the TLR7-mediated effect is most likely influenced by environmental factors rather than genetic factors.

In the current research project, we investigated two separate measurements of immune variation: in Chapter 3, we investigated self-reported respiratory infection frequency and in Chapter 4, antiviral IFNα production. The results presented suggest that the availability of the viral nucleic acid recognising receptor TLR7 is more important to prevent symptomatic respiratory infection than IFNα production *per se*, as cold frequency and IFNα production did not correlate. IFNα production is induced during viral infection, and an auto-feedback loop ensures augmented IFNα production made possible by pDC clustering (Saitoh, S-I et al. 2017). Therefore, it is possible that during asymptomatic infection, the small viral titre is not sufficient to activate the auto-feedback loop and IFNα production stays relatively controlled and does not reflect on the individual IFNα production capacity. Instead, variation in the IFNα production capacity is expected to reflect the ability to clear an infection and the severity and duration of a viral infection. High IFNα production may not necessarily be desirable, as the high IFNα production seen in auto-immune diseases such as systemic lupus erythematosus contributes to excessive inflammation. Moreover, here we focussed on TLR7 for its known major role in activating IFNα production; however variations in the signalling pathways and transcription factors of TLR7 and other IFNα-inducing PRRs can contribute to IFNα variation, and integrated signalling pathway analysis might provide a more comprehensive understanding on the modulators of antiviral immunity.

Contrasting the two groups of IFNα producers indicated that the high IFNα producer group have a better baseline status of innate immune system readiness. They upregulate many more innate immunity-related genes in comparison to the low IFNα producers. In addition to baseline *TLR7* gene expression, both *TLR8* and pDC marker *CLEC4C* gene expression were higher. The greater expression of *TLR7* and *TLR8* is unsurprising, considering that TLR7 induces IFNα production and TLR8 induced cytokines support its production. Also, as the main IFNα producer, the increased pDC numbers are expected.

Whereas high baseline *TLR7* gene expression associated with both low frequency of respiratory infections and high IFNα production, high baseline pDC quantity was associated only with high IFNα production. Instead, in Chapter 3, we observed that in men, independent of baseline *TLR7* gene expression, pDC quantity was higher in those with frequent respiratory infections. Greater pDC numbers independent of changes in *TLR7* gene expression may be a consequence of airway inflammation as is seen in asthma (Spears et al. 2011) or may be due to recent subclinical virus infection. TLR7 deficiency in asthma

patients independent of pDC numbers has been reported by others (Pritchard et al. 2014) suggesting altered TLR7 function in asthma pDCs. Another consideration for the lack of protection of respiratory infection from pDC is the role of alveolar macrophages in the lungs as important type I IFN producers during viral infection (Kumagai et al. 2007), therefore pDC would be considered to have little effect on preventing symptomatic respiratory infection. Furthermore, TLR7 expression is not limited to pDC but is known to be expressed in NK cells, eosinophils and epithelial cells and contribute to antiviral defence in those cell types.

*CLEC4C*, the pDC quantity marker, remained as one of the differentially expressed genes between high and low IFNα producers, further confirming its importance for IFNα production. Other DC related genes were also upregulated by the high IFNα producers. The complement system is a highly conserved and a central component of the innate immunity. Upregulation of the innate immune components by the high IFNα group reflected the findings of others that for a robust immune response the right innate immune components must be ready for IFNα induction (Bakker et al. 2018; Lee, MN et al. 2014).

In addition to the immunological variants, this project found several environmental and host factors that are associated with variations in antiviral immune function including asthma, obesity, sex hormones and possibly vitamin D status. Asthma and high BMI were associated with frequent colds and high BMI with low IFNα production. The associations with higher BMI were observed with only modest changes in BMI, largely within the overweight rather than obese range. The asthma group had higher BMI than controls, but the frequent colds were not confounded by the high BMI; therefore, both asthma and BMI must increase the risk of respiratory infections independently. In Chapter 3, we speculated that sex hormones in women must have a stronger influence on respiratory infection susceptibility than antiviral immune variables since both BMI and age were significantly associated with cold frequency, whereas baseline TLR7 and pDC quantity were not. As TLR7/8 genes are located on chromosome X, some genetic difference in their function has been reported (Souyris et al. 2018), however in Chapter 5 it was shown that TLR7/8 gene expression was not different between men and women in this project. The observed differential cytokine production between the genders in Chapter 3 is more likely to result from sex hormone influence or other environmental factors, rather than having a genetic basis.

Perhaps the most unexpected finding was the extent that BMI influences antiviral immunity. Surprisingly few asthma studies examining antiviral immunity take BMI into account and

report it, which might confound the interpretation of their results. Considering that asthma and BMI are correlated, to truly dissect the immune deficiency, obesity and asthma should be examined as independent variables. We observed that all participants with high BMI were more likely to have low IFNα response and females with high BMI more likely to suffer from frequent respiratory infections. The reasons for these associations may be driven by poor diet, systemic inflammation and hormonal changes in obese individuals.

In Chapter 4, the role of dietary status and nutrient-associated biochemical pathways were supported by the high BMI in the IFN-low group and the conceived link with low vitamin D and B12 availability and oxidative stress marker upregulation, which could be a result from a diet low in antioxidants. The oxidative stress response has been shown to counteract antiviral immune responses, which supports the association with low IFNα production (Mihaylova et al. 2018). In Chapter 3, high BMI was further associated with respiratory infection susceptibility. The significance of those associations is noteworthy, considering the aspect that literature has highlighted the potent ability of the gut microbiome to modify systemic immune responses (Schirmer et al. 2016). In a mouse model, the bacterial metabolite propionate was shown to protect from asthma and bronchiolitis (Lynch et al. 2018). The benefits of healthy diet and microbiome, gained in early childhood seem beneficial for protection from asthma and viral infections and are likely to continue to protect in adulthood (Lynch et al. 2017; Wood 2017).

## 6.2. Conclusion

The research described here improves the understanding of TLR7/8 and IFNα immune deficiencies in asthma, which is valuable for asthma medication development targeting respiratory infection induced asthma exacerbations.

We have shown that respiratory infections are self-reported most frequently in asthma patients and people with high BMI, which highlights those two groups at risk of symptomatic respiratory infections. Host factors such as sex, age and diet and immunological modulators, including oxidative stress and innate immune variable expression impact variation in anti-RV immune response, echoing previous studies. Modifiable lifestyle factors can be used to reduce infection-related morbidities.

Finally, genetic variation in TLR7/8 gene region affects the expression of TLR7 gene expression and due to the associations with asthma may contribute towards the deficient TLR7 expression.

In conclusion, the results here show a role for the IFNα-inducing TLR7 receptor in asthma and cold frequency. As shown in Figure 6:1, our results support that these associations are influenced by genetic variation and possibly TLR8 regulation of TLR7 and support a hypothesis of additional IFNα-independent mechanisms mediating these associations. Our results further show that the variation in immune responses is influenced by host factors such as diet, sex, age, and BMI.

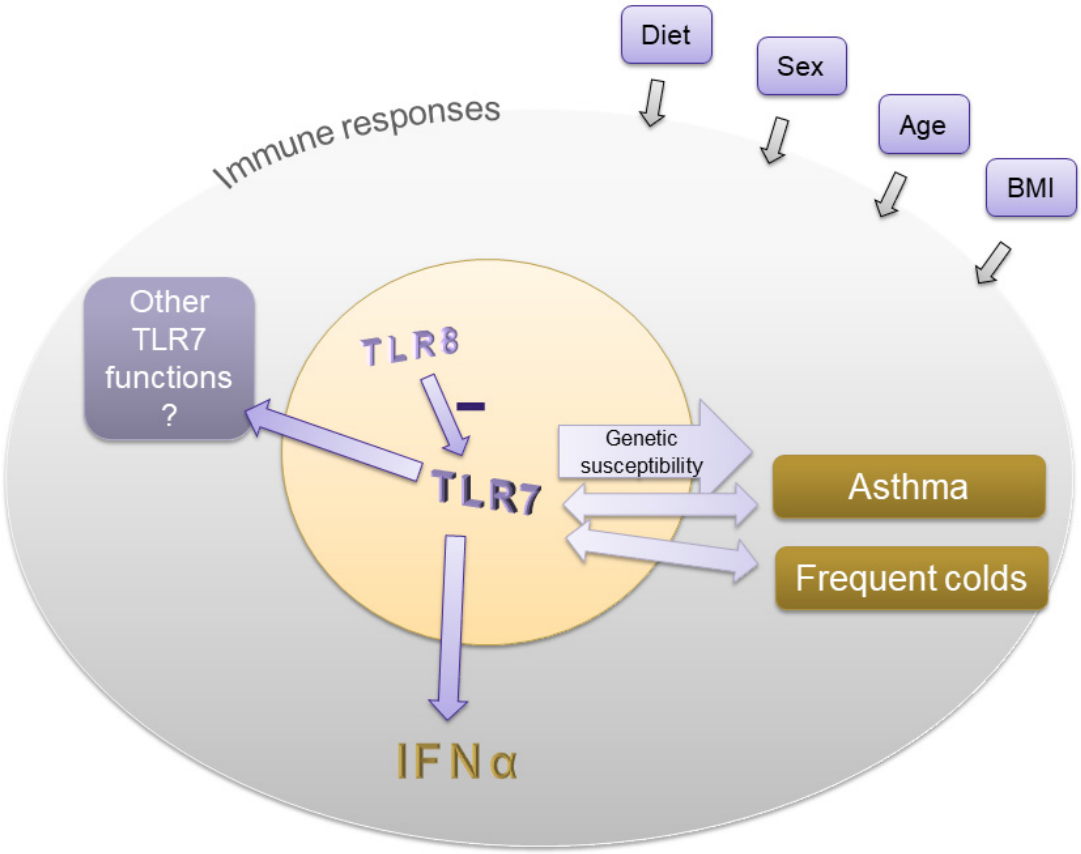


*Figure 6:1 Genetic, immunological and host factors govern asthma and antiviral immune responses mediated by TLR7 receptor.*

## 6.3. Future directions

The results reported here have raised important issues for future research. A detailed prospective study recording the respiratory infection frequency, viral titre, severity and duration will be able to address these features in an unbiased manner and confirm our findings regarding modifying immunological and host factors. We further propose that considering hormonal variation resulting from the menstrual cycle, hormonal contraceptive and replacement use, age and BMI will provide further insight into the gender disparity that we observed here in regard to the determinants of respiratory infection frequency and cytokine production.

Further research in the field of antiviral immunity in asthma should integrate information of detailed clinical parameters, inflammatory responses, and immunological factors to best describe the endotype of asthma patients who are at most risk of a deficient antiviral immune response. The work by Custovic et al. (2018), highlighted that it is still very hypothetical whether type 2 inflammation in asthma modifies antiviral immune responses or aberrant antiviral immune response predisposes to asthma, and a prospective study could best address that conundrum. Future research could also discover blood biomarkers as indicators for individuals that are a risk of hospitalisation from respiratory infection-related asthma exacerbations, and aid in developing treatments to relieve respiratory infection-related asthma symptoms, which are currently limited.

Considering the role for TLR7 in suppressing asthma inflammation, as we detected lower *TLR7* gene expression in asthma patients compared to controls *at baseline*, it is possible that either low *TLR7* gene expression is increasing type 2 inflammation in asthma, or that type 2 inflammation is reducing *TLR7* expression levels. The cross-sectional data has limited capacity to address this issue, but further research can address the causal relationships between TLR7 function and type 2 immunity in asthma with animal models and prospective studies in children. The results presented in this project suggest a pDC-independent reduction in *TLR7* gene expression in the presence of asthma that does not translate to reduced IFNα production capacity. Follow-up research should establish the TLR7 functions affected by TLR7 reduction in asthma, and if those mechanisms mediate also increased the frequency of respiratory infections in men.

Initially, we analysed only the variation in *TLR7/8* genes and their functional consequences, however in Chapter 4 we identified which genes are important for efficient IFNα response and a natural progression would be to search eQTLs and genetic variation in those genes. Additionally, perhaps the most interesting finding emerging from Chapter 5 was the association between SNP and *TLR8-AS1*. *TLR8-AS1* should be examined further to define its function, particularly in relation to TLR7 and TLR8 and whether it is associated with asthma and antiviral immune response. Previous literature suggests a TLR7-regulating role for TLR8, and it is possible that the regulation could take place in a genetic level and be mediated through *TLR8-AS1*. Further examination of TLR7 and TLR8 in co-expressing cell types can elucidate the cross-regulatory mechanisms between those receptors and the implications to their function.

Finally, although BMI was not expected to emerge as one of the key outcomes in this study, our findings indicate that further work is needed to fully understand how an increase in BMI compromises antiviral immune response and the relationship between BMI, asthma and antiviral immune response. The associations between metabolomics, oxidative stress, systemic inflammation, hormonal changes and TLR7/8 immunity in relation to BMI should be further investigated. The research field studying modifiable lifestyle factors such as diet, vitamin intake, exercise, and weight-loss and their influence on the antiviral immune response as well as asthma is well established, and the results presented herein support future research in this field.

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